EVALUATION OF PROTECTIVE EFFECT OF HYDRO-ALCOHOLIC EXTRACT OF FRUITS PEELS OF PUNICA GRANATUM LINN. AGAINST ULCERATIVE COLITIS IN RATS

Dissertation work submitted to

The Tamilnadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted by

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OCTOBER - 2017

EVALUATION CERTIFICATE

This is to certify that this dissertation work entitled **"EVALUATION OF PROTECTIVE EFFECT OF HYDRO-ALCOHOLIC EXTRACT OF FRUITS PEELS OF PUNICA GRANATUM LINN. AGAINST ULCERATIVE COLITIS IN RATS"**is the Bonafied work carried out by **NITEESH.OS, Reg. No: 261525756**under the guidance of **Prof. P. PANNEERSELVAM, M.Pharm.,** Department of Pharmacology for the partial fulfillment of the requirement of award for **Master of Pharmacy** and this is forwarded to **The Tamilnadu Dr. M.G.R Medical University, Chennai** during the academic year **2016 – 2017** has been evaluated on_____

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This is to authenticate that the plant PUNICA GRANATUM LINN of family **PUNICACEAE** have been collected from ABS Botanical Gardens, Kaaripatti, Salem district Tamil Nadu and handed over to **MR. NITEESH.OS** is studying M.Pharm, final year in **PADMAVATHI COLLEGE OF PHARMACY** for his project work.

Botanically yours,

For ABS Botanical Conservation Research and Training Centre

Executive Director

Former ***Siddha Research Counsultant*** (AYUSH)

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NITEESH.OS

DEDICATED TO MY BELOVEDFAMILY, TEACHERS AND FRIENDS

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ABSTRACT

Aim:

Evaluation of the protective effect of hydro-alcoholic extract of the fruit peels of *Punica granatum* Linn against ulcerative colitis in rats.

Methods:

Acute oral toxicity study was performed to find out the test dose according to OECD guidelines 425 and hydro-alcoholic extract of *P. granatum* fruit peel (PGPE) was found to be safe at a dose of 2000 mg/kg body weight. The acetic acid induced colitis and Trinitrobenzene sulfonic acid (TNBS) induced colitis models were used to evaluate the protective effect of extract against colitis.

Results:

The animals pretreated with hydro-alcoholic extract of *P. granatum* fruit peel (200 mg/kg and 400 mg/kg respectively) significantly restored the altered hematological, biochemical parameters to normal levels when compared with control. The protective effect of *P. granatum* peel extract was comparable to standard sulfasalazine.

Conclusion:

The findings of the present study revealed that the hydro-alcoholic extract of *P. granatum* fruit peel possessed a dose dependent significant inhibitory activity against ulcerative colitis. The results obtained established the efficacy of the *Punica granatum* fruit peel against inflammatory bowel diseases possibly by its anti-inflammatory and antioxidant properties.

Key words: Ulcerative colitis, Acetic acid, TNBS, Sulfasalazine

LIST OF ABBREVIATIONS

APCS	:	Antigen presenting cells
AOM	:	Azoxymethane
CD	:	Crohn’s disease
EBI3	:	Epstein barr virus induced gene3
EDT	:	Ethylenediamine tetra acetic acid
EGF	:	Epidermal growth factor
PGF	:	Fibroblast growth factor
GI	:	Gastrointestinal
GIT	:	Gastro intestinal tract
HETAB	:	Hexadecyl trimethyl ammonium bromide
HT	:	Hydrolysable tannins
IBD	:	Inflammatory Bowel Disease
IBS	:	Irritable Bowel Syndrom
IL	:	Interleukin
IU	:	International Units
IV	:	Intravenous
IP	:	Intra peritoneal
LPO	:	Lipid Peroxidase
Mm	:	Millimolar
MPO	:	Myeloperoxidase
NK	:	Natural killer
NO	:	Nitric oxide

NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide phosphate
PG	:	Punica granatum
PGPE	:	Hydro alcoholic extract of *Punica granatum* fruit peel
SH	:	Sulf hydryl
S.E.M	:	Standard error of mean
S.C	:	Subcutaneous
TBA	:	Thiobarbituric acid assay
TNF	:	Tumor necrosis factor
UC	:	Ulcerative colitis

INTRODUCTION

Inflammatory bowel diseases including ulcerative colitis and Crohn's disease are the most affecting human illness[1], are chronic inflammatory bowel disease[2]. Inflammatory bowel disease is an immune-mediated disease of the gastrointestinal tract caused by ulceration and inflammation[3].Crohn's disease (CD) and ulcerative colitis(UC).The pathogenesis of IBD is a multi factorial process[4]. In addition, the progress of an unusual immune and inflammatory reaction occurs, which is mediated mostly by activated neutrophils, monocytes, and macrophages and characterized by an increased development of reactive oxygen and nitrogen species. Different pro inflammatory cytokines such as interleukins (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α) chemo lines and union molecules are known to contribute to the pathogenesis of IBD[5].

Ulcerative colitis, moreover known as chronic ulceration of the intestines, is a universal disease estimated to have an incidence of 1 in 1000 persons in western countries[6]. Effective treatment relies on correct and timely diagnosis. Various factors contribute to the disease's clinical manifestations, including psychiatric and physical components, but the etiology of the disease remains poorly understood[7-8].

The majority of the current treatments for inflammatory bowel disease include treatment with glucocorticosteriods and 5-aminosalicyclic acid. Immunosuppressive drugs have also been used to heal chronic illness, regardless of the more dangerous complications and hazardous side effect associated with them[9].

Colon ulcer (ulcerative colitis) is constantly associated with changes in lipid peroxidation, Myeloperoxidase activity, catalase activity and glutathione activity.

In spite of phenomenal growth of modern medicine, there are few synthetic drugs obtainable for the treatment of ulcerative colitis. In allopathic system of treatment, corticosteroids and aminosalicylates such as sulfasalazine, mesalamine are mainly used. But these drugs have adverse effects such as head ache nausea, temporary male infertility in males etc.

Disease, decay and death have always co-existed with life, study of diseases and their treatment must also have been contemporaneous with the down of human intellect[10].

Since ancient time peoples have used herbs and its derivatives as medicines. It is a developing practice recorded in both folklore and books of early practitioners. At present, despite the abundance and improvement of synthetic drugs, an important proportion of the population of developing countries still depend on traditional medicines for their health care needs[11].

According to WHO, 60% of the world's population depend on traditional medicine, and 80% of the population in developing countries depend almost entirely on traditional medical practices, in particular, herbal medicine for their primary health care needs. The long tradition of herbal medicine continues to the present day in China, India, and many other countries[12]. Medicinal plants continue to contribute significantly to modern prescription drugs by giving lead compounds upon which the formation of new drugs can be made.

In India knowledge of medicinal herbs is very old and medicinal properties of plants are included in Rig-Veda and in Atharvaveda (3500-1500 B.C) from which Ayurved has developed in Ayurveda. The ancient well-known treatises are Charak Samhita dealing with mostly plants and Susrut Sanhita in which surgery is also mentioned. In Egypt, peoples were common with medicinal properties of plants and animals. They were familiar with human anatomy and knew of embalming the dead and preserving their bodies as described in Ebers

Papyrus (1550 B.C) an ancient book found in one of the mummies. Greek scientists contributed much to knowledge of natural history. Hippocrates (460-370 B.C) is referred to as father of medicine. Theophrastus (370-287 B.C) described medicinal plants, some of which like belladonna, ergot, opium, colchicum are used even today[13].

Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals. They are important for better health and are found naturally in variety of foods including vegetables and fruits[14].

Punica granatum Belongs to the family *Punicaceae*. It is Native to Iran; but cultivated throughout India (Jammu and Himachal Pradesh).

The plant parts contain phenolic punicalagins; Gallic acid and other fatty acids; catechin, quercetin, rutin and other flavonols; flavones, flavonones; anthocyanidins[15]. Also contains anti oxidant compounds. That can serve as primary compounds for the development of specific drugs for ulcerative colitis.

NEED AND OBJECTIVES

Need for the study:

Hepato toxicity is a comon disease and patients suffering from Various factors contribute to the disease's clinical manifestations, including psychiatric and physical components, but etiology of the disease remains poorly understood. The etiology of colitis is multifaceted.

The drugs of plant origin are gaining increasing popularity and are being investigated for remedies of varieties of diseases including hepato toxic protective activity. Ayurveda uses the plant, plant products and active ingredients present in plants for treating various diseases. Medicinal plants used for ulcerative colitis, in traditional medicine have been shown to possess promising cure from ulcerative colitis activities in animal models. There are still many more plants yet to be evaluated for above said activity.

Criteria for selection of the plant:

Number of plants being used for the treatment of hepao toxicity is known to possess antioxidants like flavonoids; alkaloids, terpenoids, etc may interact with free radicals and stop the injury caused by them.

Use of polyphenols and flavonoids is beneficial for the prevention of inflammatory disorders. pseudarthria viscida is one of such potential plant used for the treatment of ulcerative colitis disorders.

Keeping the above information in view, in the present study attempt was made to evaluate the safety and efficacy evaluation of hepato protective effect of methanolic extract of roots of pseudarthria viscida.

OBJECTIVES OF THE STUDY

1. Authentication of the plant material and preparation of Herbarium of the sample specimen.
2. To perform extraction of the dried plant material using methanol.
3. To carry out the preliminary phytochemical screening of the methanolic extract and to detect the classes of compounds present.
4. To check the in-vivo activity of the roots of Pseudarthria viscida.

I: Identification and Collection

- Identification, collection and authentication of the pseudarthria viscida root.

II: Extraction

Methanolic extract of the dried root and aerial parts of Pseudarthria vicida was taken using reflux method5gm in 30 ml for three times and the extracts were clubbed and concentrated to dryness using a rotary evaporator. The final volume was make up to 5 ml as the concentration was adjusted to 5 gm in 50 ml of methanol.

III: Preliminary phytochemical screening

- Preliminary phytochemical screening of methanolic extract.

REVIEW OF LITERATURE

3.1 The digestive system[21].

The digestive system contributes to homeostasis by breaking down food into forms that can be absorbed and used by body cells. It also absorbs water, vitamins, minerals, and eliminates wastes from the body. The food we eat contains a variety of nutrients, which are used for making new body tissues and repairing injured tissues. Food is also vital to maintain life because it is the only source of chemical energy. However, most of the food we eat contains molecules that are too large to be used by body cells. Therefore, food must be broken down into molecules that are small enough to enter body cells, a process known as digestion. The organs involved in the breakdown of food are collectively called as digestive system. Like the respiratory system, the digestive system is a tubular system. It extends from the mouth to the anus, forms an extensive surface area in contact with the external environment, and is closely associated with the cardiovascular system. The combination of extensive environmental exposure and close association with blood vessels is essential for processing the food that we eat. The medical specialty that deals with the structure, function, diagnosis, and treatment of diseases of the stomach and intestines is called gastroenterology (gastro-stomach; entero-intestines; logy-study of). The medical specialty that deals with the diagnosis and treatment of disorders of the rectum and anus is called proctology.

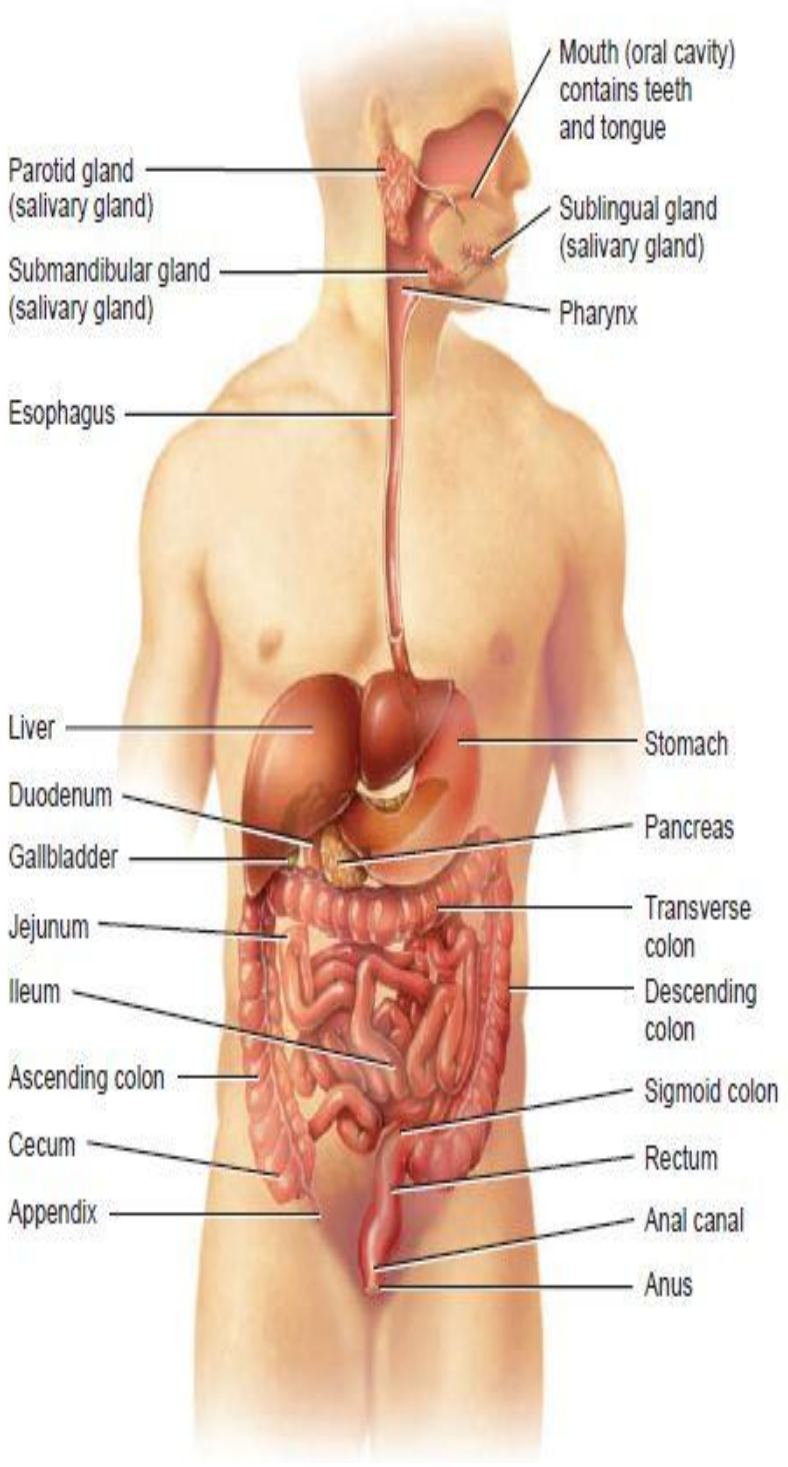


Fig 1: Right lateral view of head and neck and anterior view of trunk.

3.1.1 Anatomy of the Large Intestine [21].

The large intestine, which is about 1.5 m (5 ft) long and 6.5 cm (2.5 in.) in diameter, extends from the ileum to the anus. It is attached to the posterior abdominal wall by its mesocolon, which is a double layer of peritoneum structurally; the four major regions of the large intestine are the cecum, colon, rectum, and anal canal.

The opening from the ileum into the large intestine is guarded by a fold of mucous membrane called the ileocecal sphincter (valve), which allows materials from the small intestine to pass into the large intestine. Hanging inferior to the ileocecal valve is the cecum, a small pouch about 6 cm (2.4 in.) long. Attached to the cecum is a twisted, coiled tube, measuring about 8 cm (3 in.) in length, called the appendix or vermiform. The mesentery of the appendix, called the mesoappendix, attaches the appendix to the inferior part of the mesentery of the ileum.

The open end of the cecum merges with a long tube called the colon (food passage), which is divided into ascending, transverse, descending, and sigmoid portions. Both the ascending and descending colon are retroperitoneal; the transverse and sigmoid colon are not. True to its name, the ascending colon ascends on the right side of the abdomen, reaches the inferior surface of the liver, and turns abruptly to the left to form the right colic (hepatic) flexure. The colon continues across the abdomen to the left side as the transverse colon. It curves beneath the inferior end of the spleen on the left side as the left colic (splenic) flexure and passes inferiorly to the level of the iliac crest as the descending colon. The sigmoid colon begins near the left iliac crest, projects medially to the midline, and terminates as the rectum at about the level of the third sacral vertebra.

The rectum, the last 20 cm (8 in.) of the GI tract, lies anterior to the sacrum and coccyx. The terminal 2–3 cm (1 in.) of the rectum is called the anal canal. The mucous membrane of the anal canal is arranged in longitudinal folds called anal columns that contain a network of

arteries and veins. The opening of the anal canal to the exterior, called the anus, is guarded by an internal anal sphincter of smooth muscle (involuntary) and an external anal sphincter of skeletal muscle (voluntary). Normally these sphincters keep the anus closed except during the elimination of feces

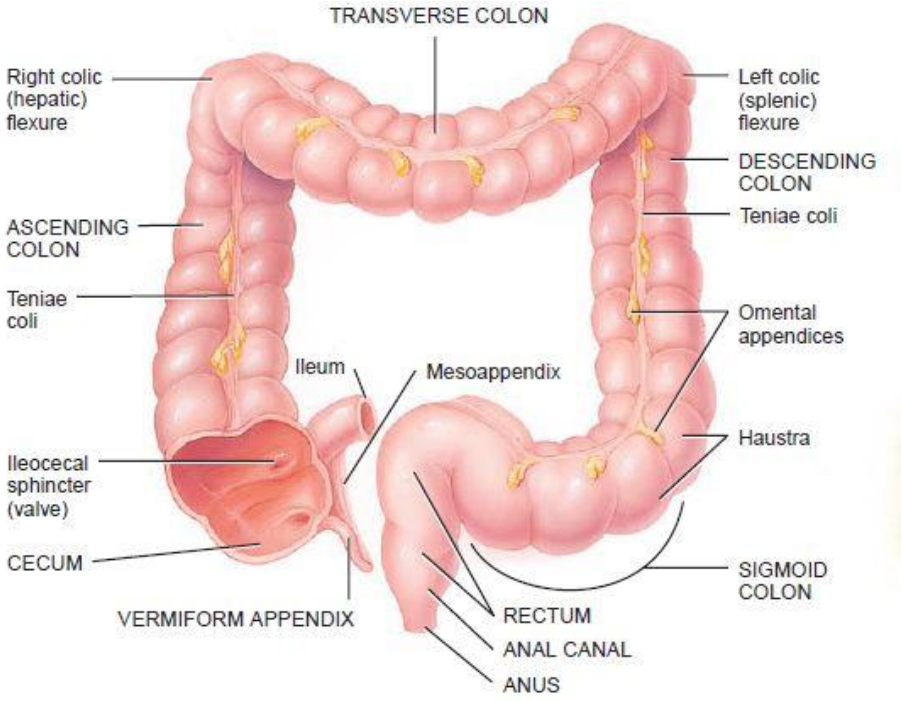


Fig 2: Anterior view of large intestine showing major regions.

3.1.2 The colon[22].

The colon (also called the large bowel) is an organ of the gastrointestinal tract. The job of the colon is to absorb fluids, to form stools, and to eliminate stools. The colon is a hollow tube surrounded by muscles. It measures about 5 feet long and 2.5 inches in diameter. It has a moist interior lining that protects nerves located in the colon wall.

These nerves send and receive messages to the brain about the colon contents. The brain then directs the appropriate colonic action. Fluids and dietary fiber help to maintain colon health.

Parts of the Colon, Movement of Waste & Stools

Several parts make up the continuous tube of the colon. Each part contributes to the movement of materials and the formation of stools. The parts include: Ileocecal Valve: The ileocecal valve is a fold of mucus membrane at the entry way to the colon. It is located where the small intestine meets the colon. Materials from the small intestine pass into the colon through this valve. Vermiform Appendix: The appendix is attached to the bottom of the cecum. This is a twisted coiled tube that is about 3 inches long. The function of the appendix is not known.

Cecum: The cecum is a kind of reservoir to receive fecal materials as they enter the colon. The cecum is located below the ileocecal valve at the base of the colon. The upper part of the cecum is open to the colon. The muscles of the cecum and the colon advance feces upward out of the cecum.

Ascending Colon: The ascending colon is located on the right side of the abdomen above the cecum. Here, most of the water is absorbed from the feces as it moves upward through the ascending colon. The ascending colon "ends" at the hepatic flexure where the colon bends to the left and connects to the transverse colon.

Transverse Colon: The transverse colon runs laterally across the abdomen below the belly button. As feces move across the transverse colon, stools begin to take form. The transverse colon "ends" at the splenic flexure where the colon bends again and connects to the descending colon which heads down the left side.

Descending Colon: The descending colon runs down the left side of the abdomen. Stools move down the descending colon. Stools are now more solid in form. Here, stools may be stored for a time. The descending colon "ends" where it continues into the sigmoid colon.

Sigmoid Colon: The sigmoid colon angles to the right, curving down and inward to about the midline, then it curves slightly upward where it connects to the top of rectum. Stools continue their descent as they move through sigmoid colon. Stools may also be stored here for a time before they are moved into the rectum.

Processing & Activity in the Colon

Aided by enzymes and muscular action, the mouth, stomach and small intestine perform their individuated jobs of breaking down and absorbing nutrients. The liquid that these organs generate is called chyme. This liquid waste matter is called feces. It is passed to the colon for further processing and elimination. In the colon, instead of the enzymatic action that occurs in other organs of the G.I. tract, further breakdown of fecal matter and the production of substances occur by way of bacterial fermentation. Cellular exchanges, bacteria, and muscular actions all play a part in processing the feces as it passes through the colon:

Fluid Absorption: The colon lining contains epithelial cells that absorb fluids and other substances such as vitamins and electrolytes. It is the absorption of fluids and bacterial processing that transforms the soupy fecal matter into a stool.

Secretion of Mucous: The colon lining contains epithelial cells that secrete mucus. This mucus moisturizes and lubricates the colon lining. This lining protects the colon wall and nerve tissues.

Bacterial Growth: Bacteria live and grow along the colon lining. Using the fluids and foods you intake, bacteria actually manufacture the nutrients that sustain their environment and their food supply.

Manufacture of Some Vitamins &Electrolytes: Bacteria change proteins into amino acids and break these amino acids down further into indole and skatole (which gives stools their odor), hydrogen sulfide, and fatty acids. Bacterial action also synthesizes some vitamins (K

and some B), electrolytes, and breaks down bilirubin into a pigment that gives stools their brown color.

Production of Lubrication: Bacteria ferment soluble fiber into a lubricating gel that is incorporated into the stool mass as it is formed. This gel helps to make stools soft and flexible. Some of this gel also coats the exterior of the stools and is used by the colon to moisturize the colon lining. This lubrication helps to ease stool passage through the colon.

Defense against Infection: Healthy intestinal bacteria help to groom the colon and keep it clean so that infections do not develop. They also help to fight the growth of infectious bacteria.

Stool Formation: To form stools, muscles in the colon churn the soupy liquid fecal matter as fluids are extracted until the particles have the consistency to form a stool.

3.2 Inflammatory bowel diseases

3.2.1 Disease description

Inflammatory bowel disease (IBD) is a range of chronic idiopathic inflammatory intestinal situation. IBD causes significant gastrointestinal symptoms that include diarrhea, abdominal pain, bleeding, anemia, and weight loss. IBD also is connected with a spectrum of extra intestinal manifestations, including arthritis, uveitis, iritis. IBD mainly divided into two major subtypes:

- Ulcerative colitis
- Crohn's disease

Ulcerative colitis is mainly characterized by mucosal inflammation of the colon starting at the anal verge and extending proximally for a variable extent. Crohn's disease, is characterized

by transmural inflammation of any part of the gastrointestinal tract but most commonly the area adjacent to the ileocecal valve[23].

Patients with both Ulcerative Colitis and Crohn's disease have long been reported to have a high risk for colorectal cancer[24]. Although etiology of ulcerative colitis is unknown, but the current study suggests that multiple immune, genetic, and environmental factors influence both the initiation and multiplication of colitis. There is evidence for an intense local immune response along with recruitment of lymphocytes and macrophages followed by discharge of soluble cytokines and other inflammatory mediators. Subsequent activation of these cells causes a self-augmenting cycle of cytokine production, cell recruitment and inflammation.

This uncontrolled immune system activation results in a sustained high production of cytokines such as tumor necrosis factor (TNF)-α and interleukins (IL-1β and IL-8). In addition to cytokines, leukotrienes, thromboxane, platelet-activating factor, nitric oxide and reactive oxygen species are also released from activated mucosal cells[25].

In ulcerative colitis, only the colon is affected; of the multiple layers of the intestinal wall, only the innermost lining of the colon, the mucosa, becomes inflamed in ulcerative colitis patients. Ulcerative colitis also spreads proximally, meaning it starts from the rectum and can spread continuously to the rest of the large intestine (colon).

3.2.2 Epidemiology

In regions with a high prevalence of IBD, such as North America, the incidence was seen to increase between the 1960s and 1980s, reaching the current plateau[26–28]. In the United States specifically, an estimated 1 million individuals have IBD[29], with about 30,000 new cases reported each year. The incidence is evenly divided between UC and CD[29].

The peak age of onset for IBD is 15 to 30 years old, although it may occur at any age. About 10% of cases occur in individuals, 18 years old. Both UC and CD have a bimodal age distribution, with a second, smaller peak occurring in individual's ages 50 to 70 years[27, 29]. Ulcerative colitis is somewhat slightly more common in males, whereas CD is marginally more frequent in women (female-to-male ratio range, 1:1 to 1.8:1).Both disorders are tend to occur in higher socioeconomic groups[26, 27]. Breakdowns by racial and ethnic subgroups indicate that higher rates of IBD occur in people of Caucasian and Ashkenazic Jewish origin than in individuals from other backgrounds[26, 30]. The distribution of IBD among ethnic and racial groups remains dynamic. In past decades, it was thought that IBD occurred less frequently in ethnic or racial minority groups compared with whites. This gap has been closing, with an increased incidence in African Americans [26] and in second generation south Asians who have migrated to developed countries[26, 31].

Most patients are able to live normal, productive lives. Each year in the United States, IBD accounts for 700,000 physician visits, 100,000 hospitalizations, and disability in 119,000 patients. Over the long-term, up to 75% of patients with CD and25% to 33% of those with UC will require surgery[31].

3.2.3 ETIOLOGY

It is likely that a number of factors contribute to the development of mucosal inflammation. Also, variations in influence may account for the clinical diversity seen in UC and CD[32]. Although the exact cause of ulcerative colitis remains undetermined, the condition appears to be related to a combination of genetic and environmental factors. Whole genome scans have found susceptibility genes for UC on chromosomes 1 and 4, although these loci have not been uniformly confirmed[33].

Whilst it is unfortunately true to say that the etiology of ulcerative colitis remains obscure, during the past few decades many attempts have been made to unravel this complicated problem. The most popular theories concerning the etiology of ulcerative colitis can be listed as follows.

Infection

Even though, ulcerative colitis had clearly been separated from the contagious forms of diarrhea by the middle of the nineteenth century, until recently many workers refused to believe that this disease was not infectious in nature. Perhaps the most widely celebrated of these was Bargen, who in 1924 claimed to have isolated a diplococcus from the stools of patients suffering from ulcerative colitis-and even produced a vaccine against this diplococcus, which was claimed to be effective in such patients[34]. Unfortunately in the fullness of time it became apparent that these claims were not entirely justified. Bargen's 'diplococcus' was shown to be almost certainly a harmless type of enterococcus found in the stools of vast numbers of the general population; and no real convincing evidence was forthcoming to suggest that the vaccine was effective in preventing attacks of colitis.

Since that date other authors have postulated that a number of organisms might be partly responsible for ulcerative colitis, including parasites, fungi and various viruses (Fradkin, 1937[35]: Dragstedt, Dack & Kirsher, 194[36]; Henderson, Pinkerton & Moore, 1942[37]; Victor, Kirsner & Palmer, 1950[38]).

Unfortunately, however, further careful controlled studies have failed to uphold the claims of these various organisms to be the offending agents in causing ulcerative colitis. Thus there is little concrete evidence nowadays in support of any hypothesis proposing an infectious etiology for ulcerative colitis.

Mucinases

In 1947 Meyer and his colleagues proposed that ulcerative colitis might be due to destruction of the mucus lining the surface of the colon by enzymes, (which were termed mucinases), thus rendering the colon more susceptible to attack by bacterial and other agents. It was shown that stool concentration of lysozyme (an enzyme claimed to be capable of digesting colonic mucus), was higher in colitic patients than in normal controls, and that the stool concentration rose and fell during exacerbations and relapses of colitis[39].

It remained; however, far from certain that lysozyme was the cause of this disease; since the changes observed in lysozyme titre could very well have been the result instead. Finally this hypothesis fell into disrepute when it was shown (albeit in vitro) that lysozyme was incapable of dissolving or digesting human mucus[40].

Allergy

Despite the fact that some workers have succeeded in producing a type of delayed hypersensitivity reaction in the colon of the experimental animal (Rosenberg & Fischer, 1964;

Bicks & Rosenberg, 1964), the position of allergens in the etiology of ulcerative colitis is also somewhat uncertain at the present time[41] .

The idea that cow's milk might be in some way responsible for the development of ulcerative colitis was first emphasized by Andresen (1925, 1942)[42].

More recently as a result of studies by Truelove and his colleagues at Oxford, several additional facts have come to light which appear to support this hypothesis. These workers have shown that occasional patients with ulcerative colitis experience a remission of their

disease when milk products are excluded from their diet, and suffer a relapse when they are re-introduced.

Also it has been shown that the titre of antibodies to milk proteins in the circulation is significantly raised in colitic patients when compared with normal matched controls, and it has been further suggested that a significantly greater proportion of colitis patients have abandoned breast feeding in the 1st month of life than healthy matched controls[41-45].

Auto immunity

The first studies suggesting that ulcerative colitis might be an autoimmune disease are widely attributed to Broberger & Perlmann (1959)-although Cornelis (1958) had already suggested such a possibility. Broberger & Perlmann (1959), using an extract of foetal colon in tissue culture, were able to show haemagglutinating antibodies to the colonic mucosa in no less than twenty out of thirty children with ulcerative colitis[46].

It was still possible to argue that the changes which Broberger & Perlmann had observed were occurring as a totally independent phenomenon, and were unrelated to the disease process of ulcerative colitis. But this argument was in part refuted by their further studies (Perlmann & Broberger, 1963) showing that the leucocytes from patients with ulcerative colitis had a cytotoxic effect upon the foetal colon cells in tissue culture, an effect which was inhibited by pre-treatment with colon antigen[47].However, recent studies by Harrison (1965)[48] and by Wright & Truelove (1966) have shown that autoantibody to colon can be demonstrated in only 15 or 20% of patients with ulcerative colitis[49] ; and moreover there is little correlation between the clinical course of colitis and the incidence of circulating antibodies to colon.

Genetic Factors

Epidemiological and family studies demonstrate that genetic factors play a role in the susceptibility to IBD[50]. The disease is, however, genetically complex and cannot be explained by a single gene model alone[50]. It is thought that UC and CD may be heterogeneous polygenic disorders sharing some but not all susceptibility loci.

Most likely, the disease phenotype is determined by several factors, including interaction between allelic variants at a number of loci, as well as genetic and environmental influences[50]. There have been a number of reports of families in which several members have inflammatory bowel disease and it seems likely that there is a genetic predisposition to these conditions but the magnitude of the risk is uncertain. Binder et a144 reviewed 152 patients with ulcerative colitis compared with matched controls of the same social class. In eight families a relative was affected compared with only one in the control group. These findings have been confirmed in Stockholm[51].

Familial and ethnic syndromes

There is an increased prevalence of IBD in first- and second-degree relatives and a higher relative risk among siblings. The familial frequency of IBD ranges from 20% to 30% in referral-based studies and between 5% and 10% in population surveys[52]. The higher risk for IBD in the Jewish population [53] suggests that genetic factors may play a larger role in some subgroups[50]. In families with a high incidence of IBD among first degree relatives, 75% of those affected are concordant for either UC or CD, whereas 25% are not concordant, with some members having UC and others having CD[52]. This finding indicates that multiple, overlapping genetic factors may contribute to disease pathogenesis. Further support for a genetic susceptibility comes from the finding of an association between IBD and other syndromes with a genetic predisposition[50].

PSYCHOLOGICAL FACTORS

In 1930, a young medical student, Cecil D. Murray, published a notable paper which claimed that psychological factors might be important in the etiology of ulcerative colitis. Psychological and physiological evidence has been adduced in favour of this hypothesis.

Many psychiatrists have observed that a large percentage of patients suffering from ulcerative colitis can be induced to confess to 'interhuman conflicts' in the immediate period before an attack of the disease (Murray, 1930; Sullivan, 1935; Palmer, 1948; Paulley, 1950, 1956; Groen and Van der Valk, 1956)[53]. Further workers, using the Rorschach and other tests, have claimed that patients with ulcerative colitis are infantile, dependent, passive, egocentric, hesitant, and uncertain. Changes in the attitude to life undoubtedly occur in patients during severe attacks of ulcerative colitis. Patients become depressed, morose, and dependent upon their clinical attendants.

Environmental Triggers

‘‘Westernization’’

Ulcerative colitis is most prevalent in developed regions, including the United States, United Kingdom, and Scandinavia[26, 27]. The higher incidence of UC seen in industrialized countries and the dramatic increase in cases during the 20th century supports the theory that environmental factors contribute to disease development[28]. This may also account for the north-to-south variation and higher frequency in urban communities compared with rural areas. Interestingly, increases in incidence have recently been noted in southern countries and Asia and among migrants to first-world countries [26, 27]. It is postulated that this is the result of ‘‘westernization’’ of lifestyle, such as changes in diet, smoking, and variances in exposure to sunlight, pollution, and industrial chemicals[26].

Tobacco smoking

The strongest environmental risk factor for IBD is tobacco smoking, particularly among consumers of cigarettes. The relationship between IBD and smoking is complex, suggesting unique pathophysiological factors for both UC and CD. Numerous case-control studies have shown that current smoking is protective against UC (relative risk, 40% of that of non-smokers), with results that are consistent across diverse geographic regions[28].The decreased risk for UC in smokers appears to be dose dependent[54]. Current smoking also is protective against sclerosing cholangitis[55, 56].and pouchitis[57]. Paradoxically, ex-smokers are approximately 1.7 times more likely to develop UC than those who never smoked[28]

Diet

Studies seeking to link diet and IBD are generally inconclusive. There is some evidence that a higher intake of fatty acids increases the risk for IBD[28]. Similarly, Persson *et al*[58]. Suggests that frequent fast-food intake confers a 3- to 4-fold greater risk for IBD.

3.2.4. Pathophysiology

Ulcerative colitis is a chronic idiopathic inflammatory disorder of the GI tract. While Crohn's disease and ulcerative colitis share a number of gastrointestinal and extraintestinal manifestations and can respond to a similar array of drugs, emerging evidence suggests that they result from fundamentally distinct pathogenetic mechanisms[59]. Histologically, the transmural lesions in Crohn's disease exhibit marked infiltration of lymphocytes and macrophages, granuloma formation, and submucosal fibrosis, whereas the superficial lesions in ulcerative colitis have lymphocytic and neutrophilic infiltrates. Within the diseased bowel in Crohn's disease, the cytokine profile includes increased levels of interleukin-12, interferon-g, and tumor necrosis factor-a, findings characteristic of T- helper 1-mediated inflammatory

processes. In contrast, the inflammatory response in ulcerative colitis resembles more closely that mediated by the TH2 pathway.

The innate immune system is the body's nonspecific defense against pathogens; it responds immediately or within the first few hours after a challenge. This is commonly considered the first line of defense and includes such physical barriers as the skin and the intestinal mucosa as well as immune cells that identify and remove foreign bodies. The innate immune system reacts to the chemical properties of the antigen rather than to the specific antigen itself. The acquired immune system, however, responds specifically to antigens. The antigen is processed and recognized, and immune cells that are specific to that antigen are then selectively proliferated. Memory is also a part of adaptive immunity, which improves the efficiency of future immunologic responses. The traditional view of the pathogenesis of inflammatory bowel disease is that intestinal inflammation is mediated by cells of the acquired immune system. The chronic inflammation could result from overly aggressive activity of effectors lymphocytes and proinflammatory cytokines, which overcome the control mechanisms.

Alternatively, IBD may result from a primary failure of regulatory lymphocytes and cytokines, such as interleukin-10 and transforming growth factor to control inflammation and effector pathways[60, 61]. In addition, a central pathogenic mechanism in Crohn disease is the resistance of T cells to undergoing apoptosis after activation.

Presentation of intraluminal antigens to mucosal lymphocytes by antigen-presenting cells (APCs) leads to the generation of effector responses. In the normal gut (left), overt inflammation is prevented by controlling the activation of mucosal effector T cells (Teff) through at least 2 distinct mechanisms (**Fig.3**). First, regulatory T-cell subpopulations (Treg) in the mucosal immune system suppress effector T-cell activity in part through the production

of interleukin-10 and transforming growth factor. Second, control is also provided by eliminating Teff by apoptosis, thereby preventing undesired overexpansion. In individuals with IBD, both of these regulatory mechanisms seem to be defective (right).

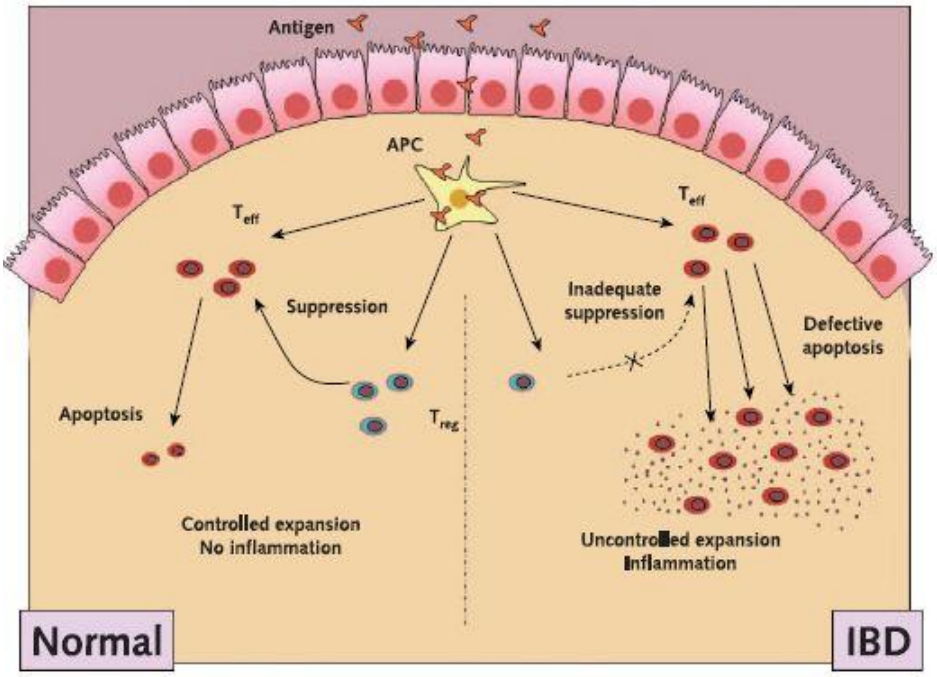


Fig. 3: The traditional paradigm for the pathogenesis of inflammatory bowel disease (IBD).

The exact cause of this phenomenon has not yet been fully elucidated; nonetheless, the ability of anti–tumor necrosis factor and anti–interleukin-12 antibodies to efficiently prevent or reverse clinical and experimental IBD is largely mediated by their ability to restore mucosal homeostasis and redirect mucosal effector T cells into apoptotic pathways. In both scenarios, lymphocytes are considered to be the major culprits. However, there is emerging evidence that defects in the innate immune system play an equal or even more important role in IBD[62, 63]. Evidence of the role of the innate immune system comes from the recently discovered association between Crohn disease and loss-of-function mutations in the caspase-activating and recruitment domain 15 gene (card15 - so named because the protein it encodes contains a

CARD protein–protein interaction domain), which is also known as nod2. The NOD2 protein is an intracellular receptor for a component of the bacterial cell wall and plays an important role in triggering cells of the innate immune system.

3.2.5 ULCERATIVE COLITIS: MACROSCOPIC FEATURES

The macroscopic assessment of colectomy specimens remains important despite the widespread use of colonoscopy and endoscopic biopsy in IBD diagnosis. A careful and thorough description, ideally with gross photographs, can be critical in assigning the correct diagnosis. Factors that are particularly important to note are:

- The **distribution** of colitis.
- Appearance of **ulcers** and intervening mucosa, and
- The appearance of the **colonic wall**.

Ulcerative colitis begins in the rectum and extends proximally. Disease confined to the rectum and associated with prominent lymphoid follicles has been regarded as a separate disorder by some (follicular proctitis[64]) based on reduced responsiveness to therapy. Apparent rectal sparing can occur due to prior topical steroid therapy and skip lesions in the appendix and cecum are allowable. Crohn's disease can affect any part of the Inflammatory Bowel Disease colon and may produce a pan colitis, but the ascending colon and rectum are the most common colonic sites.

In colectomy, performed for severe UC the ulcers can have several forms. Typically, broad and flat areas of ulceration due to mucosal undermining and loss are seen between obviously inflamed, hyperaemic and granular mucosa, often with pseudo polyp formation. Intermittent ulceration can occur giving the impression of skip areas[65] and in these cases histology is necessary to confirm milder colitis (rather than normal mucosa) between the ulcerated areas.

The second type of ulceration seen in severe UC is linear, occurring where the plicae semilunares converge along the lines of attachment of the three taeniae coli. This should not be confused with the fissuring ulceration of CD. Finally, fine fissuring into submucosa occurs in toxic dilatation, although this is better appreciated microscopically. The ulcers of CD, on the other hand, vary from small aphthous ulcers aligned longitudinally and surrounded by normal mucosa to deep and narrow, fissuring ulcers separating mounds of normal appearing mucosa. This latter appearance is termed cobble-stoning. In the ileum, fissuring is most prominent on the mesenteric side of the bowel wall compared with the antimesenteric location in ischaemic enteritis.

The colonic wall is not significantly thickened in UC. There may be some shortening and simplification of the haustral pattern due to fibrosis in the mucosa and upper submucosa as a result of repeated flares. Although generally a mucosal disease, UC can show limitedtransmural inflammation in areas of severe ulceration, resulting in serosal hyperemia. This occurs in the absence of wall thickening or fat creeping.

In CD the colonic wall is typically thickened due to edema, fibrosis and smooth muscle proliferation. This thickening and structuring is sometimes so severe as to suggest malignancy macroscopically. The serosal fat is increased and wraps around the intestinal wall. Some authors have advocated the separation of Crohn's disease into perforating and non-perforating types on the basis of perceived differences in clinical aggressiveness and complications but this subdivision remains clinically controversial[66].

Fig 4: Pan-ulcerative colitis

Mucosa has a lumpy, bumpy appearance because of areas of inflamed but intact mucosa separated by ulcerated areas.

3.2.6 ULCERATIVE COLITIS: MICROSCOPIC FEATURES

Normal mucosa

Before discussing the salient microscopic features of inflammatory bowel disease, it is necessary to outline the normal appearances of mucosa and submucosa. The crypts within the mucosa are of uniform length, diameter and spacing giving an appearance that has been likened to test tubes in a rack. The crypts should reach the muscularis mucosa in all areas of the colon except the rectum, where it is normal to have uniform mild shortening. In the region just above the anorectal junction, significant crypt distortion is common and this area is generally avoided at biopsy. The crypt density is 7-8 per mm except in areas of lymphoid follicle formation where the density reduces significantly. Branched crypts can be seen in normal biopsies but should not exceed 10% of all crypts; from a practical point of view more than two branched crypts in a biopsy is excessive[67].

The lamina propria contains a mixed inflammatory infiltrate in which lymphocytes and plasma cells predominate, neutrophils are particularly rare and eosinophils may be present in variable numbers. Inflammatory cells are generally more numerous in the superficial lamina propria. Lymphoid aggregates can be present in the mucosa and upper submucosa,

particularly in the rectum, and they may cause the mucosa to bulge slightly. Macrophages containing mucin (muciphages) are commonly seen in normal rectal biopsies, and are increased nonspecifically following mucosal injury. The normal submucosa is composed of loose fibrovascular connective tissue. The thickness is not uniform, and areas of relative thinning occur where the mucosa almost touches the muscularis propria. If this area becomes inflamed it may be misinterpreted as fissuring ulceration[67].

Idiopathic IBD is generally diagnosed in the presence of abnormal mucosal architecture with or without superimposed inflammation in the lamina propria. The guidelines outlined by the British Society of Gastroenterology are currently the favoured ones for histological diagnosis.

The typical mucosal architectural changes develop several weeks after the onset of the first attack of colitis so that biopsies taken very early in the course, or in children[68], may not be diagnostic. Architectural changes take about 15 days to develop but the basal lymphoplasmacytosis occurs earlier. There is a variable degree of crypt shortening; reduced crypt density, variable crypt diameter and crypt branching that can be subtle, particularly in quiescent colitis.

The pattern of mucosal inflammation is often distinctive in active IBD. In many cases the inflammatory infiltrate is predominantly composed of lymphocytes and plasma cells distributed throughout the lamina propria, often with a prominent basal lymphoplasmacytosis. Neutrophils are also prominent and are associated with cryptitis and crypt abscess formation.

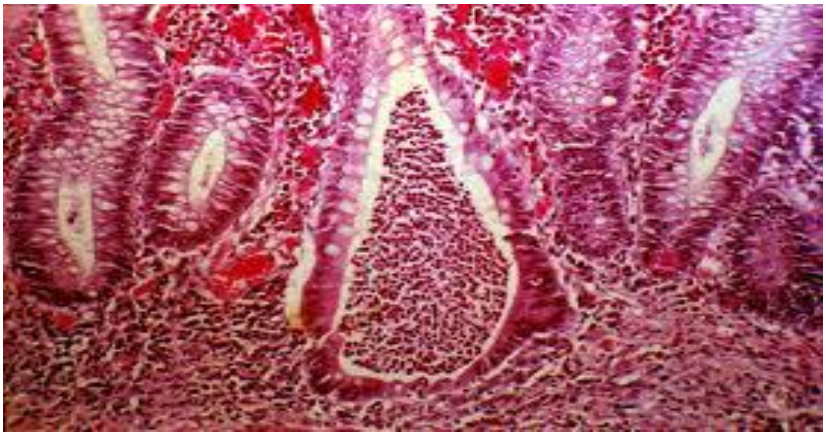


Fig .5: Characteristic findings of IBD in a case of ulcerative colitis: crypt distortion, cryptitis and crypt abscess.

Rupture of large crypt abscesses with undermining of the adjacent mucosa may be seen, or large abscesses can rupture into the upper submucosa. Non-caseating epithelioid granuloma can be seen in some but not all cases of CD, but it must be remembered that a granulomatous reaction is relatively common around ruptured crypts in UC and this patternis not diagnostic of CD.

Because of the size and nature of mucosal biopsies, fissuring ulceration and transmural inflammation cannot be diagnosed reliably from usual-sized biopsies, but fissuring may be suspected if granulation tissue is seen running down one side of a biopsy which has relatively unremarkable mucosa at the other end.

3.3 TREATMENT

AMINOSALICYLATES

Aminosalicylates can be used in combination with steroids to induce and maintain remission in patients with inflammatory bowel disease. The first-line therapy for mild to moderate UC generally involves mesalamine (5-aminosalicylic acid, or 5-ASA). The archetype for this class of medications is sulfasalazine, which consists of 5-ASA linked to sulfapyridine by an azo bond. Although this drug was originally developed as a treatment for rheumatoid

arthritis, clinical trials serendipitously demonstrated a beneficial effect on the gastrointestinal symptoms of subjects with concomitant UC.

Sulfasalazine is most effective at maintaining remission in UC. When it reaches the colon, the diazo bond is cleaved by bacterial azoreductase, liberating mesalamine and sulfapyridine. Sulfapyridine is absorbed and metabolized by hepatic acetylation or hydroxylation followed by glucuronidation. Given individually, either 5-ASA or sulfapyridine is absorbed in the upper gastrointestinal tract; the azo linkage in sulfasalazine prevents its absorption in the stomach and small intestine, and the individual components are not liberated for absorption until colonic bacteria cleave the bond. 5-ASA is the active therapeutic moiety; sulfapyridine contributes little to the therapeutic effect.

Although mesalamine is a salicylate, its therapeutic effect does not appear to be related to cyclooxygenase inhibition; indeed, traditional nonsteroidal anti-inflammatory drugs may actually exacerbate IBD. Many potential sites of action have been demonstrated *in vitro* for either sulfasalazine or mesalamine, including inhibition of IL-1 and TNF- α production, inhibition of the lipooxygenase pathway, the scavenging of free radicals and oxidants, and inhibition of NF- *ķ*B (nuclear factor kappa B), a transcription factor pivotal to the production of inflammatory mediators[69].

Although it is not active therapeutically, sulfapyridine causes many of the same side effects observed in patients taking sulfasalazine. To preserve the therapeutic effect of 5-ASA without the side effects of sulfapyridine, several second-generation 5-ASA compounds have been developed. They are divided into two groups: prodrugs and coated drugs. Prodrugs contain the same azo bond as sulfasalazine but replace the linked sulfapyridine with either another 5-ASA (olsalazine) or an inert compound (balsalazide).

These compounds act at similar sites along the gastrointestinal tract as sulfasalazine. The alternative approaches employ either a delayed-release formulation or a pH-sensitive coating. Delayed-release mesalamine is released throughout the small intestine and colon, whereas pH-sensitive mesalamine is released in the terminal ileum and colon. The different distributions of these drugs following delivery have potential therapeutic implications.

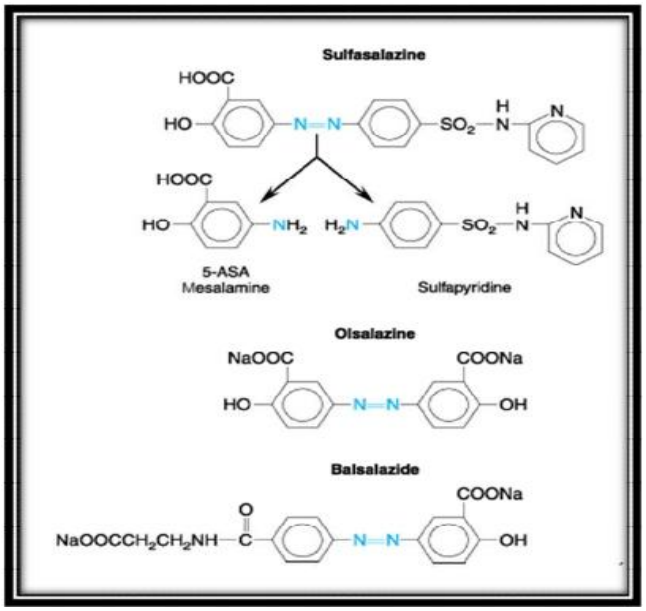


Fig 6: Structures of sulfasalazine and related agents.

Oral sulfasalazine is of proven value in patients with mild or moderately active ulcerative colitis, with response rates in the range of 60% to 80%[70]. The usual dose is 4 g/day in four divided doses with food; to avoid adverse effects, the dose is increased gradually from an initial dose of 500 mg twice a day. High doses as 6 g/day can be used but that can cause an increased incidence of side effects.

Topical preparations of mesalamine suspended in a wax matrix suppository (ROWASA) or in a suspension enema (CANASA) are effective in active proctitis and distal ulcerative colitis, respectively[71].They appear to be superior to topical hydrocortisone in this setting, with response rates of 75% to 90%. Mesalamine enemas (4 g/60 ml) should be used at bedtime

and retained for at least 8 hours; the suppository (500 mg) should be used two to three times a day with the objective of retaining it for at least 3 hours.

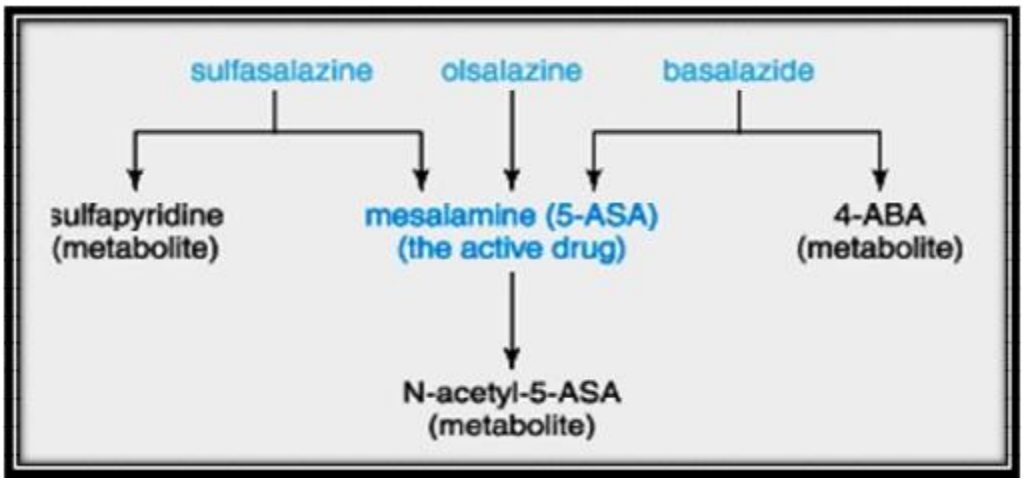


Fig 7: Metabolic fates of the different oral formulations of mesalamine (5-ASA).

GLUCOCORTICOIDS

The glucocorticoid properties of hydrocortisone and prednisolone are the mainstay of IBD treatment. The preferred steroid is prednisolone, administered orally, rectally or parenterally in emergency situations. Corticosteroids can be used either alone or in combination with a suitable mesalamine formulation to induce and maintain remission in inflammatory bowel disease. The incidence of adverse effects appears to increase when prednisolone doses are higher than 40 mg/day. An alternate-day regimen is helpful because it reduces adrenal suppression. Azathioprine is a purine analogue that competitively inhibits the biosynthesis of purine nucleotides. Its mode of action is not well understood. Once absorbed azathioprine is almost entirely metabolized to 6-mercaptopurine[72].

Initial dose for prednisone is between 40 to 60 mg per day; higher doses are rarely more effective[73]. In severely ill hospitalized patients, 100 mg of hydrocortisone administered intravenously every eight hours is a reasonable initial therapy. Intravenous therapy generally produces a rapid improvement of symptoms, with maximal benefits occurring when the

corticosteroid has been administered for six to eight days. Once the patient's symptoms have improved, prednisone is tapered by 5 to 10 mg per week, until the dosage reaches 15 to 20 mg per day. This dosage is then tapered by 2.5 to 5 mg per week until the drug is discontinued. The goal is to remove patients from corticosteroids within a relatively short period of time while maintaining disease remission. Concomitant use of 5-ASA agents can be helpful. Alternatively, long-term, alternate-day corticosteroid therapy can be used in patients with refractory CD, although it may be necessary to use dosages of 20 to 25 mg every other day[74].

Budesonide is an enteric-release form of a synthetic steroid that is used for ileocecal CD[75]. It is thought to deliver adequate steroid therapy to a specific portion of the inflamed gut while minimizing systemic side effects caused by extensive first-pass hepatic metabolism to inactive derivatives. Topical therapies (e.g., enemas and suppositories) are also effective in treating colitis that is limited to the left side of the colon. Budesonide (9 mg/day for 10 to 12 weeks) is effective in the acute management of mild-to moderate exacerbations of CD, but its role in maintaining remission has not been fully determined[76.]

Hydrocortisone also can be given once or twice daily as a 10% foam suspension that delivers 80 mg hydrocortisone per application; this formulation can be useful in patients with very short areas of distal proctitis and difficulty retaining fluids.

IMMUNOSUPPRESSANTS

Several drugs initially developed for cancer chemotherapy or as immunosuppressive agents in organ transplants have been adapted for the treatment of IBD. Immunosuppressant drugs can be an invaluable adjunct therapy for the treatment of patients with intractable inflammatory bowel disease or complex, inoperable perianal disease. Although immunosuppressant agents have significant side effects, they are safer and better tolerated

than long-term corticosteroid therapy. Before immunosuppressant therapy is initiated, side effects and other treatment alternatives should be discussed with the patient. At this stage, it is best to set a definable goal, uch as closure of a fistula or tapering the patient off of corticosteroids, and a minimum three-month time frame should be set to reach that goal[72].

Since the early 1970s, azathioprine and mercaptopurine have been used to treat IBD. These drugs are superior to the placebo, but their full effects may not become apparent for as long as three months. Azathioprine and mercaptopurine are beneficial in 50 to 70% of patients with intractable perianal CD[77].

The cytotoxic thiopurine derivatives mercaptopurine (6-MP) and azathioprine are used to treat patients with severe IBD or those who are steroid-resistant or steroid-dependent[78].

Both are prodrugs; azathioprine is converted to mercaptopurine, which is subsequently metabolized to 6-thioguanine nucleotides, typically, azathioprine is administered at a dose of 2 to 2.5 mg/kg and mercaptopurine is given at a dose of 1.5 mg/kg. Because of concerns of side effects, these drugs were used initially only in CD.

For both azathioprine and mercaptopurine, the initial dosage is 50 mg per day. A therapeutic benefit usually occurs at dosages of 50 to 100 mg per day for mercaptopurine and 75 to 150 mg per day for azathioprine.

Mild leukopenia suggests that the drug is effective and therefore more likely to benefit the patient. It is prudent to obtain a complete blood count every two weeks during the initial treatment phase in patients with active disease and every three months in patients on maintenance therapy[79].

For more than 20 years, low-dose methotrexate therapy has been used in patients with intractable psoriasis and rheumatoid arthritis. Methotrexate was engineered to inhibit dihydrofolate reductase, thereby blocking DNA synthesis and causing cell death. First used in cancer treatment, methotrexate was subsequently recognized to have beneficial effects in autoimmune diseases such as rheumatoid arthritis and psoriasis. The anti-inflammatory effects of methotrexate may involve mechanisms in addition to its inhibition of dihydrofolate reductase. One study showed that this treatment was beneficial in 70% of patients with severe IBD[80].

CYCLOSPORINE

The calcineurin inhibitor cyclosporine is a potent immunosuppressant drug used in organ transplantation. Since the mid-1980s, this drug has also been used to treat patients with IBD. At this time, cyclosporine is most useful in severely ill patients with UC who have not responded to corticosteroid therapy. In such patients, intravenously administered cyclosporine is highly effective for rapid disease control, and it may allow patients to avoid surgery. However, after one year, 70 to 80% of these patients may still require surgery. Thus, in many patients, the role of cyclosporine is to change a risky emergency operation into a less urgent procedure[81].

Cyclosporine is effective in severe UC that has failed to respond adequately to glucocorticoid therapy. Between 50 and 80% of these severely ill patients improve significantly (generally within 7 days) in response to intravenous cyclosporine (2 to 4 mg/kg daily), which sometimes allows them to avoid an emergent colectomy.

3.4 Different Animal Models

ACETIC ACID INDUCED ULCERATIVE COLITIS IN RATS:

The experiment was performed using acetic acid for inducing colitis[82]. All the animals will be pre-treated with the respective drugs for 8 days along with the normal diet. On the 4th day of the treatment, the animals will be fasted overnight with access to glucose containing water ad libitum. On the 5th day after 1 h of the aforementioned treatments, the animals (Groups II to V) will be anesthetized by ether inhalation and a polypropylene tube with 2 mm diameter will be inserted through the rectum into the colon to a distance of 8 cm. Initially, each rat received a 1 ml saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Then, a solution of 2 ml of acetic acid (3%, v/v) in 0.9% saline will be instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 s to prevent the leakage of the intracolonic instillate. After 72 h of single dose administration of acetic acid (on 8th day), clinical activity scores will be measured and the animals will be anaesthetized with ether and blood will be collected by retro orbital puncture for biochemical estimation. The animals will be sacrificed by cervical dislocation and colon will be dissected out. Colon will be flushed gently with saline and weighed. It is used for macroscopic scoring, histopathological and biochemical estimations[83].

In the intestinal tract, energy status is a fundamental regulator of epithelial cell metabolism[84]. An energy deficit has been considered to be a pathogenic factor in ulcerative colitis, which is substantiated by the fact that the intestinal mucosa has a limited capacity for-denovo synthesis of purine nucleotides[85], and is more prone to reduced ATP concentrations compared with the liver or muscle[86].

On the other hand, IL-6 and tumor necrosis factor α (TNF-α) has been shown to play an important role in the pathogenesis of inflammatory bowel disease[87]. These pro-inflammatory

cytokines drive the activation and recruitment of inflammatory cells, amplify the production of other pro-inflammatory cytokines, and activate nuclear transcription factors, thereby promoting and maintaining the inflammatory response[88]. Additionally, release of transforming growth factor- α (TGF-α) and expression of TGF- α mRNA are increased after acute gastric injury and in the colonic mucosa from patients with IBD[89, 90].

DSS induced colitis

For the induction of colitis, mice were administered 3% (w/v) DSS dissolved in drinking water for various time periods. One cycle consisted of 7 days of DSS treated water followed by 14 days of normal drinking water. Animals were divided into various groups, each group consisting of 8 animals; and mild, moderate and severe colitis were induced in them. Group 1 received normal drinking water and served as control. Group 2 (mild colitis) received DSS (3%, w/v) for 7 days and the animals were sacrificed on the 8th day. Group 3 (moderate colitis) received DSS (3%, w/v) from days 1 to 7 and 22 to 28 and the animals were sacrificed on the 29th day. During the remission period (days 8–21) mice were administered normal drinking water. Similarly, group 4 (severe colitis) received DSS (3%, w/v) from days 1 to 7, 22 to 28 and 43 to 49 and the animals were sacrificed on the 50th day. During the remission period (days 8–21 and 29–42) mice were administered normal drinking water. To assess the extent of colitis, weight loss, stool consistency and rectal bleeding were monitored daily in order to calculate the disease activity index (DAI). DAI is referred to as the average combined score of weight loss (0–4), stool consistency (0–4) and rectal bleeding (0–4) used to score clinical symptoms[91].

Feeding mice for several days with DSS polymers in the drinking water induces a very reproducible acute colitis characterized by bloody diarrhea, ulcerations and infiltrations with

granulocytes. It is believed that DSS is directly toxic to gut epithelial cells of the basal crypts and therefore affects the integrity of the mucosal barrier.

As T- and B-cell deficient mice also develop severe colitis, the adaptive immune system obviously does not play a major part in this model . Hence, the acute DSS colitis model is particularly useful to study the contribution of innate immune mechanisms of colitis. In addition, the DSS model has been shown to be suitable to study epithelial repair mechanisms. Studies with TLR4−/− and MyD88−/− mice suggest that TLR signaling is required to limit bacterial translocation after DSS induced intestinal epithelial injury suggesting that TLR signaling is important for the maintenance of the epithelial barrier . In susceptible strains, the administration of DSS for several cycles (e.g., 7 days DSS, 14 days water) results in chronic colitis and if combined with a single initial dose of the genotoxic colon carcinogen azoxymethane (AOM), in inflammation-associated colorectal cancer. Patients with UC have an increased risk for the development of colon cancer. As colonic inflammation is suggested to play a key role in IBD-related colorectal cancer, the AOM/DSS model is a very useful tool to study mechanisms linking inflammation to colon carcinogenesis.

TNBS induced colitis

Colitis can be induced in susceptible mouse strains by intrarectal instillation of the haptenating substances TNBS/ DNBS or Oxazolone dissolved in ethanol with or without a skin presensitation step. Ethanol is required to break the mucosal barrier, whereas TNBS/Oxazolone is believed to haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. As CD4+ Tcells have been shown to play a central role in chronic TNBS colitis, this model is useful to study T helper cell-dependent mucosal immune responses. The TNBS colitis model has been very useful in studying many important aspects of gut inflammation, including cytokine secretion patterns,

mechanisms of oral tolerance, cell adhesion and immunotherapy. Murine TNBS colitis has been initially described in SJL/J mice, a mouse strain with high susceptibility that develops chronic TNBS colitis characterized by a predominant TH1-mediated immune response with dense infiltrations of lymphocytes/macrophages and thickening of the colon wall . However, studies with IFN-γ−/− mice on a Balb/c background showed that in these mice TNBS colitis may be associated with a TH2-mediated colonic patch hypertrophy. In SJL/J mice, Oxazolone colitis has been shown to affect only the distal colon and particularly mucosal layers.

Histological features and an elevated production of TH2 cytokines (IL-4, IL-5 and IL-13) of unstimulated and αCD3/αCD28-stimulated lamina propria T cells are in these mice, in some aspects, similar to characteristics that have been observed in human UC. In contrast to several other murine colitis models, treatment with neutralizing anti-IL-4 antibodies or a decoy IL-13Rα2-Fc protein ameliorates disease. This group has shown that Oxazolone colitis depends on the presence of IL- 13 producing invariant natural killer (NK) T cells. Consistently, mice deficient for Epstein Barr virus induced gene 3 (EBI3) that have a defect in iNKT cell generation, are resistant to Oxazolone colitis. Thus Oxazolone colitis is one of the few models suitable to study the contribution of the TH2-dependent immune response to intestinal inflammation. As in the TNBS model, oxazolone colitis is strain dependent and requires individual optimization. This is exemplified by the fact that oxazolone at lower doses can induce a mixed TH1/TH2-dependent colitis[92].

Indomethacin-induced colitis in rats

The experiment was performed using indomethacin. Normal or untreated animals which did not receive any treatment; Control animals received only indomethacin (7.5 mg/kg, s.c.), on two consecutive days; Drug treated animals received 7 days pre-treatment with polyherbal

formulation and indomethacin (7.5 mg/kg, s.c.) on 8th and 9th day. Drug treatment was continued till 11th day; Prednisolone treated group, which received prednisolone (2 mg/kg, p.o., for 4 days) and indomethacin (7.5 mg/kg, s.c., for 2 days). Prednisolone and indomethacin treatment was started on the same day. Rats of either sex (200–250 g) were given indomethacin 7.5 mg/kg subcutaneously on two consecutive days. On the fourth day animals were sacrificed by cervical dislocation and dissected open to remove GIT (from stomach to anus). GIT was flushed gently with saline and cut open. Cecum, 10 cm long pieces of ileum and colon were scored for inflammation based on their macroscopic features. Tissues were fixed in 10% formalin saline and examined histopathologically. Quantification of inflammation was done using myeloperoxidase assay.

Iodoacetamide-induced colitis

This model, initially described by Satoh, Sato, Takami and Szabo, is based on the fact that endogenous sulfhydryl (SH) compounds, such as glutathione, play an important role in the protection of gastric mucosa. Instillation of SH blocker in the colon could induce colitis and cause injury to the mucosa by decreasing the amount of defensive SH compounds. After the induction of UC, many changes characteristic of inflammation were taken into account to determine the degree or severity of the disease related to the administered doses of iodoacetamide. The different alterations included diarrhea, dilatation, adhesion, mucosal damage (varying from slight mucosal erosion to deep lesion), and inhibition of body weight gain.

Heparin, a polyanionic highly sulfated linear polysaccharaide, belonging to the family of glycosaminoglycans, has been implicated in the management of UC. Heparin therapy resulted in significant improvement in macroscopic and microscopic features of colitis, accompanied by a partial reduction in myeloperoxidase (MPO) levels.

The regular trends in the behavior of colonic fibroblast growth factor (FGF)- binding activity and HB–epidermal growth factor (EGF)-binding activity levels were reversed with heparin therapy. However, conflicting studies and results have been published on the response to heparin in patients with severe UC since it was first reported. The investigators concluded that one mechanism of heparin-mediated improvement in colitis may involve tissue healing associated with changes in functional levels of colonic growth factors. On the other hand, using the same model, it was shown that anti-surfactant-like particles antibodies ameliorated the inflammation in IBD.

Oxazolone colitis:

Boirivant, Fuss, Chu, and Strober reported that an enema of oxazolone with ethanol would induce colitis. In comparison with TNBS, this agent caused colitis earlier. The peak of body weight loss and diarrhea was seen on the second day after the enema, and symptoms diminished after 10–12 days. Colitis, accompanied by ulcers, was localized in the distal colon. Histopathological studies showed that the numbers of epithelial cells, goblet cells, and glands have decreased compared with controls. In contrast to TNBS colitis, these findings closely resembled those of UC.

Peptidoglycan–polysaccharide (PG–PS) colitis

In 1988, Sartor, Bond, and Schwab demonstrated that the intramural injection of the bacterial cell wall component PG–PS into the distal colon of rats induced transmural enterocolitis. In genetically susceptible Lewis rats, chronic granulomatous colitis developed 3–4 weeks after injection. Histopathologically, there were thickening of the colon wall, infiltration of lymphocytes, macrophages, and neutrophils. PG–PS increased mucosal permeability and MPO activity, and enhanced NO production and collagen synthesis. Data obtained from this model clearly showed that the cell wall components of non-pathogenic resident enteric

bacteria are sufficient to induce acute and chronic colitis in a susceptible host when they penetrate the colon wall[93].

3.5 PLANT LITERATURE

3.7.1 Detail of plant is given below:

Name of the plant: *Punica granatum Linn.*

Family: *Punicaceae.*

Habitat: *Native to Iran; but cultivated throughout India.*

Vernacular Names:

English - Pomegranate.

Ayurvedic - Daadima, Daadimba, Raktapushpa, Dantabijaa, Raktakusumaa, Lohitpushpaka.

Unani - Anaar, Roomaan, Gulnaar,Gulnaar Farsi

Siddha/Tamil - Maathulai.

Hindi: Anar

Unani - Anaar, Roomaan, Gulnaar,Gulnaar Farsi.

Marathi: Dalimb

Manipuri: Kamphoi

3.7.2 Plant description:

Order: Myrtales

Kingdom: Plantae

The pomegranate is an ancient fruit that has not changed much throughout the history of man. It was found in the Indus Valley so early that there is a word in Sanskrit for pomegranate. The pomegranate is also significant in Jewish, Christian and Muslim traditions. The

pomegranate is native of Iran and Afghanistan, known in ancient Egypt. This includes only two species. More than 500 cultivars of Punica granatum exist with specific Characteristics such as fruit size, exocarp and aril color, etc. Originating from the Middle East, pomegranate is now widely cultivated throughout the world, and also widely consumed. Pomegranate has been used for centuries in the folk medicine of many cultures[94].

Fig 8: Photograph of plant *Punica granatum* Linn

- **Fruits**: is a berry of 5 to 12 cm diameter with a leathery, deep red peel (husk, rind, and pericarp are synonyms). The fruit's interior is separated by membranous walls into compartments containing arils filled with pulp. Each aril contains one angular seed. Phenolic Compounds are present in different parts of pomegranate plants; they are found in seeds, arils, fruit peels, leaves, flowers, tree bark, and roots[94].

Fig 9: Photographs showing fruits of *Punica granatum* Linn.

- **Leaves:** leaves are opposite or sub opposite, glossy, narrow oblong, entire, 3-7 cm long and 2 cm broad[94].

Fig 10: Photograph showing leaves of *Punica granatum* Linn.

- **Seeds:** Each seed has a surrounding water-laden pulp-the edible aril-ranging in colour from white to deep red. The seeds are embedded in a white, spongy pulp.

Fig 11: Photograph showing seeds of *Punica granatum* Linn.

- **Flowers:** are bright red, 3 cm in dm, with 4 or 5 petals.

Fig 12: Photographs showing flowers of *Punica granatum* Linn.

Chemical constituents:

Pomegranate Juice - anthocyanins, glucose, ascorbic acid, ellagic acid, Gallic acid; caffeic acid; catechin, EGCG, quercetin, rutin; numerous minerals, particularly iron; amino acids.

Pomegranate seed oil - 95-percent punicic acid; other constituents, including ellagicacid; other fatty acids; sterols.

Pomegranate pericarp (Peel, rind) - Phenolic punicalagins; gallic acid and other fatty acids; catechin, EGCG; quercetin, rutin and other flavonols; flavones, flavonones; anthocyanidins.

Pomegranate leaves - Tannins (punicalin and punicafolin); and flavones glycosides, including luteolin and apgeni.

Pomegranate flower - Gallic acid, ursolic acid; triterpenoids, including maslinic and Asiatic acid; other unidentified constituents

Pomegranate roots and bark - Ellagitannins, including punicalin and punicalagin; numerous piperidine alkaloids.

Table 1: Plant component and its constituents

Plant component	Constituents
Pomegranate juice	Anthocyanins, glucose, ascorbic acid, gallic acid, caffeic acid, catechin, rutin, amino acids.
Pomegranate seed oil	Purinic acid, ellagic acid, sterols, fatty acids.
Pomegranate peel	Gallic acid, catchin, quercetin, rutin, flavonoids.
Pomegranate leaves	Tannins, flavones glycosides.
Pomegranate flower	Gallic acid, ursolic acid, triterpenoids, asiatic acid
Pomegranate roots	Ellagitannins, piperidine alkaloids.

The major class of pomegranate phytochemical is polyphenols that predominate in fruits[95].Pomegranate polyphenols include flavonoids condensed tannins and hydrolysable tannins. Hydrolysable tannins (HTs) are found in the peels (rind, husk, or pericarp), membranes and piths of the fruit. HTs are predominant polyphenols found in pomegranate juice and account for 92% of its antioxidant activity[96].

The presence of phytoconstituents make the plant useful for treating different ailments and have a potential of providing useful drugs of human use. In the present study, we have found that most of the biologically active phytochemicals were present in the ethanolic, aqueous

and chloroform extracts of *Punica granatum* peel, whole fruit and seeds. The medicinal properties *of Punica granatum* peel, whole fruit and seeds extract may be due to the presence of above mentioned phytochemicals.

3.7.3 Medicinal Uses:

Bark and the roots are believed to have anthelmintic and vermifuge properties; the fruit peel has been used as a cure for diarrhea, oral aphthae, and as a powerful astringent, the juice as a blood tonic, and the flowers as a cure for diabetes mellitus[94]. Consumption of polyphenoles and flavonoids is beneficial for the prevention of cardiovascular, inflammatory and other diseases[96]. Rind of fruit is astringent, stomachic, digestive. Used for diarrhea, dysentery, colitis, dyspepsia and uterine disorders. Leaf is used in stomatitis .Fresh juice of fruit is refrigerant, antiemetic; given as An adjuvant in diarrhea, dyspepsia, biliousness, inflammations of the stomach, palpitation, excessive thirst and fevers[97]. Bark of stem and root—anthelmintic, febrifuge, given for night sweats. Rind of fruit, bark of stems and root is antidiarrhoeal. Powdered flower buds are used in bronchitis[20].

MATERIALS AND METHODS

4.1 Materials

4.1.1 Collection and authentication of plant material:

The fresh peels of *Punica Granatum* used for the present studies were collected from Kariapatti, Salem in April 2017. It was authenticated by A.Balasubramaniyam, Executive Director, ABS Botanical Garden, Salem. The dried peels were pulverized separately into coarse powder by a mechanical grinder and were used for extraction.

Methods:-

4.2 Preparation of Hydro Alcoholic Extract:

The powdered material was subjected to hydro-alcoholic extraction in Soxhlet apparatus by using water and ethanol as solvents. The powdered material (150 gm) of *P. granatum* peels were packed in Soxhlet extractor and extracted using solvents for 15hrs successive cycles. The temperature was maintained on an electric heating mantle with thermostat control. Appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The extract was concentrated by using rotary flash evaporator. The concentrated extract was then air dried at room temperature, weighed and percentage yield was calculated. The color and consistency of the extract were noted.

4.3 Preliminary Qualitative Phytochemical Analysis: [98, 99]

The following tests were carried out on the herbal extract to detect various phytoconstituents.

A. Test for Alkaloids:

0.5 gm extract was dissolved in 10 ml of dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids:

I. Mayer's test

To one ml of filtrate, 2 ml of Mayer's reagent was added in a test tube. Formation ofYellow cream precipitate indicates the presence of alkaloids.

II. Wagner's test

One ml of filtrate was treated with few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

III. Dragendorff's test

One ml of filtrate was treated with few drops of Dragendorff's reagent. Formation of reddish brown precipitate indicates the presence ofalkaloids.

IV. Hager's test:

One ml of filtrate was treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids.

B. **Tests For Glycosides**

I. **Bromine water test:**

Test extract was dissolved in bromine water. Formation of yellow precipitate indicates the presence of glycosides.

II. **Baljet test**:

Test extract was treated with sodium pirate.Formation of yellow to orange colourindicates the presence ofglycosides.

III. **Keller-Killiani test**:

0.5g of dried extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solutions. This was then under laid with 1 ml of concentrated H2SO4. It forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green, indicates the presence ofglycosides.

IV. Legal's test:

Test extract was treated with pyridine (made alkaline by adding sodium nitroprusside solution).Formation of pink to red colour indicates the presence ofglycosides.

C. **Tests ForTannins**

I. **Ferric chloride test**:

Few drops of 5% w/v FeCl3 solution was added to 1-2ml of the extract.Formation of brown colour indicates the Presence of pseudo tannins.

II. Vanillin hydrochloride test:

Extract was treated with vanillin hydrochloride reagent. Formation of purplish red colour indicates the Presence of tannins.

III. Gelatin test:

Extract was treated with gelatin solution.Formation of white precipitate indicates the Presence of tannins.

D. Tests For Saponins

I. Sulphur test:

Sulphur was added to the extract solution.Sulphur sinks at bottom indicates the Presence ofsaponins.

II. Froth's test:

The extract was diluted with distilled water and shaken for 15 min.Formation of foamindicates the Presence ofsaponins.

III. Liebermann Buchard's test:

The extract was treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. The formation of brown ring at the junction indicated the presence of steroidal saponins.

E. Tests For Carbohydrates

Extracts were dissolved individually in 5ml of distilled water and filtered. The filtrates were used to test the presence of carbohydrates.

I. Molisch's Test:

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml concentrated sulphuric acid was added carefully along the sides of the test tubes. Formation of violet ring at the junction indicates the presence of carbohydrates.

II. Benedict's Test:

Filtrates were treated with Benedict's reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.

III. Fehling's Test:

Filtrates were hydrolyzed with dilute hydrochloric acids, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate indicates the presence of reducing sugars.

IV. Barfoed's test:

1ml Barfoed's reagent was added to 1ml of extract and heat for 2 min.Formation of red precipitate indicates the presence of carbohydrate.

V. **Seliwanoff's test:**

Plant Extract was treated with Seliwanoff's reagent and heat strongly.Formation of a characteristic cherry red colour indicates the presence of keto sugar.

F. **Tests For Flavonoids**

I. Lead acetate test:

Lead acetate solution was added to small amount of extract.Formation of yellow precipitate indicates the presence of flavonoids.

II. Shinoda test:

A little quantity of extract was dissolved in alcohol with few fragments of Mg turnings and concentrated HCl drop wise.Formation of pink or crimson-red colourindicates the presence of flavonoids.

III. Alkaline reagent test:

Increasing amount of sodium hydroxide was added to the sample extract. Formation of yellow colouration observed which disappears upon addition of acid indicates the presence of flavonoids.

IV. Ferric chloride test:

Extract was treated with ferric chloride solution.Formation ofIntense green to black colourindicates the presence of flavonoids.

G. Tests For Steroids

I. **Salkowski reaction:** 2mg of dry extract was shaken with $CHCl_3$, to the $CHCl_3$ layer, H_2SO4 was added slowly by the sides of testtube.Formation of red colourindicates the presence ofsteroids.

II. **Lieberman Burchard's test:** 2mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1ml of conc. H_2SO_4.Formation ofred violet or green colour indicates the presenceofSteroids.

4.4 Experimental animals:

Healthy Wistar albino rats (150–200g) of either sex were used for the experiment were procured form the animal house of Padmavathi College of pharmacy, Dharmapuri. They were maintained under standard conditions (temperature 22 ±2oC, relative humidity 60 ± 5% and 12 hr light/dark cycle). The animals werehoused in sanitized polypropylene cages containing sterile paddy husk as bedding.They had free access to standard pellet diet and water ad libitum. All the animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals"prepared by the "National Academy of Sciences" and published by the "National Institute of Health".

The hydro-alcoholic extract of *P.granatum* peels were subjected to the following Investigations:

Pharmacological activities:

1. Toxicity studies (LD50)
2. Ulcerative colitis protective activity

Determination of Acute toxicity (LD_{50}):[100, 101]

The hydro alcoholic extract of the leaves of*Punica granatum* Linn was dissolved in water to prepare a dose of 2000mg/kg b.w. of animal and administered 1ml/100g b.w. ofthe animal. Acute toxicity study of the extractwas done according to acute toxic classic method (OECD guideline 425, 2006) using albino female rats to determine the safe dose.

Procedure:

Female Albino rats weighing between 200-300g were used to carry out acute toxicity studies. By applying limit test method, the animals were given hydro-alcoholic extract of Punica granatum fruit peel in water at a dose of 2000mg/kg of body weight orally. The animals were continuously observed for 2-3 hrs for general behavioral, neurological, autonomic profile and death for a period of 24 hrs and there after once daily for 14 days. The following check list (Irwin's table) was employed for the acute toxicity studies:

1. **Behavioral Profile**

   Awareness: alertness, stereotypy, visual placing

   Mood: grooming, restlessness, fearfulness

2. **Neurological profile**

   Motor activity: spontaneous activity, touch response, pain response,

   Tremor, gait, grip strength, pinna reflex, corneal reflex.

3. **Autonomic profile:**

Writhing,defecation, urination, piloerection, heart rate, respiratory rate.

Selection and preparation of dose for pharmacological screening:

There was no mortality and sign of toxicity at a limit dose of 2000mg/kg. Hence for evaluation of protective activity against colitis, two dose levels were selected in such a way that the lower dose (200mg/kg) was one tenth of the maximum dose and a high dose (400mg/kg), which was twice that of one tenth doses during acute toxicity studies for the ulcerative colitis protective activity.

ULCERATIVE COLITIS PROTECTIVE ACTIVITY

Chemicals and instruments

All chemicals and solvents used in the study were of analytical grade. The ulcerative colitis inducing chemicals acetic acid (Agappe suppliers) and tri nitro benzene sulfonic acid (himedia suppliers) and other chemicals like Nitroblue tetrazolium, Phenazine methasulphate, NADH, Thiobarbituric (Himedia suppliers), used for the present study were obtained from Agapee distributers.

Instruments like U.V (Shimadzu), Micro centrifuge (REMI), rotary flash evaporator (Superfit, Rotavap), and Microscope (Magnus), were used for the present study.

ACETIC ACID INDUCED ULCERATIVE COLITIS IN RATS [63]:

EXPERIMENTAL DESIGN:

Healthy albino rats of the either species weighing 150–200 g, will be used in the study and were divided into four groups with five animals in each group (n=6) as follows

- Group I (Vehicle control) – 1 ml distilled water.
- Group II (Experimental control) – Toxic control (2 ml acetic acid)
- Group III (Standard) – Sulfasalazine (100 mg/kg, p.o.)+ 2mlAcetic acid

- Group IV (Test Low Dose) - PGPE (200 mg/ kg) + 2ml Acetic acid
- Group V (Test High Dose) - PGPE (400 mg/ kg) + 2ml Acetic acid

The experiment was performed using acetic acid for inducing colitis[82]. All the animals were pre-treated with the respective drugs for 8 days along with the normal diet. On the 4th day of the treatment, the animals werefasted overnight with access to glucose containing water ad libitum. On the 5th day after 1 h of the aforementioned treatments, the animals (Groups II to V) wereanesthetized by ether inhalation and a polypropylene tube with 2 mm diameter were inserted through the rectum into the colon to a distance of 8 cm. Initially, each rat received a 1 ml saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Then, a solution of 2 ml of acetic acid (3%, v/v) in 0.9% saline wereinstilled into the lumen of the colon and maintained in a supine trendelenburg position for 30 s to prevent the leakage of the intracolonic instillate. After 72 h of single dose administration of acetic acid (on 8th day), clinical activity scores weremeasured and the animals wereanaesthetized with ether and blood werecollected by retro orbital puncture for biochemical estimation. The animals weresacrificed by cervical dislocation and colon weredissected out. Colon wereflushed gently with saline and weighed. It is used for macroscopic scoring, histopathological and biochemical estimations[83].

TRINITROBENZENE SULFONIC ACID (TNBS) INDUCED ULCERATIVE COLITIS IN RATS.

EXPERIMENTAL DESIGN:

Healthy albino rats of the species weighing 150–200 g, will be used in the study and were divided into five groups with five animals in each group (n=6) as follows.

- Group I (Vehicle control) – 1 ml distilled water.
- Group II (Experimental control) – Toxic control (TNBS 1 ml)
- Group III (Standard) –Sulfasalazine (100 mg/kg, p.o.)+ TNBS
- Group IV (Low dose) - PGPE (200 mg/ kg) + TNBS
- Group V (High dose) - PGPE (400 mg/ kg) + TNBS

The experiment was performed using TNBS for inducing colitis. All the animals will be pre-treated with the respective drugs for 8 days along with the normal diet. On the 4th day of the treatment, the animals will be fasted overnight with access to glucose containing water ad libitum. On the 5th day after 1 h of the aforementioned treatments, the animals (Groups II to V) will be anesthetized by ether inhalation and a polypropylene tube with 2 mm diameter will be inserted through the rectum into the colon to a distance of 8 cm. Initially, each rat received a 1 ml saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Then, a solution of 1 ml of TNBS (dissolved in 1.0 ml 50% ethanol) will be instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 s to prevent the leakage of the intracolonic instillate. After 72 h of single dose administration of TNBS (on 8th day), clinical activity scores will be measured and the animals will be anaesthetized with ether and blood will be collected by retro orbital puncture for biochemical estimation. The animals will be sacrificed by cervical dislocation and colon will be dissected out. Colon will be flushed gently with saline and weighed. It is used for macroscopic scoring, histopathological and biochemical estimations[92].

Biochemical parameters estimated includes

1. Reduced glutathione (GSH)
2. Lipid peroxidation (MDA)
3. Myeloperoxidase (MPO) activity
4. Catalase (CAT)

Preparation of colon homogenate

The colon was quickly removed and perfused immediately with ice-cold saline(0.9% NaCl). A portion of the liver was homogenized in chilled Tris-HCl buffer(0.025 M, pH 7.4) using a homogenizer. The homogenate obtained wascentrifuged at 5000rpm for 10 min, supernatant was collected and used foranalysis.

BIOCHEMICAL ESTIMATION:

a) Estimation of reduced glutathione (GSH)[102]

Procedure

1g of scrapped tissue of the colon was homogenized with 10ml of 10% TCA in icecold condition and centrifuged at 3000rpm for 10 min. 2ml of phosphate buffer ofpH 8 was added to 0.5ml of supernatant. Followed by 0.2ml of DTNB solutionand colour intensity of yellow colour was measured immediately at 412nmagainst blank. Increase in absorbance (optical density) is directly proportional toconcentration of GSH and vice versa and % increase in GSH was calculated.

b) Estimation of lipid peroxidation (MDA)[103]

Procedure

Lipid peroxidation was estimated in terms of thiobarbituric acid reactivespecies (TBARS). One ml of the colon homogenate was added to 2.0ml of theTBA-TCA-HCl reagent. The contents were boiled for 15 min, cooled and centrifuged at 10000 rpm to remove the precipitate. The Absorbance of supernatantwas determined against blank. Colour intensity of pink colour chromogen wasmeasured at 535nm. Increase in the Absorbance indicates greater MDA anddecrease in Absorbance indicates lesser MDA indirectly showing lesser lipidperoxidation. Hence the Abs was measured and % inhibition of lipid peroxidationwas calculated.

c) Estimation of myeloperoxidase (MPO)[104]

Procedure

Pieces of inflamed tissues (colon-4 cm) were taken. The tissue was then rinsedwith ice-cold saline, blotted dry, weighed and excised. Minced tissue washomogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4), usingRemi tissue homogenizer (RQ-127A). The homogenate was centrifuged at 3500 rpmfor 30 min at 4◦C (Remi centrifuge C23). The supernatant was discarded. 10 ml of ice-cold 50mM potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and l0mM EDTA was then added to thepellet. It was then subjected to one cycle of freezing and thawing and brief period (l5s) ofsonication. After sonication solution was centrifuged at 15,000 rpm for 20 min. Myeloperoxidase(MPO)activitywasmeasuredspectrophotometrically as follows. 0.1 ml of supernatant was combined with 2.9 ml of50mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride and0.0005% H2O2.

The change in absorbance was measured spectrophotometrically(Shimadzu UV 1 60A UV-VIS spectrophotometer), at 460 nm. One unit of MPOactivity is defined as the change in absorbance per minute by 1.0 at room temperature,in the final reaction.

d) **Estimation of catalase(CAT)**

Procedure

The reaction mixture (1.5ml, vol.) contained 1.0ml of phosphate buffer, 0.1ml oftissue homogenate (supernatant) and 0.4ml of H_2O_2. The reaction was stopped bythe addition of 2.0ml of dichromate-acetic acid reagent and mixture was heatedfor 2 min. Colour intensity of green colour was measured calorimetrically at 620nm. Increase in Absorbance (optical density) is directly proportional toconcentration of CAT and % increase in CAT was calculated.

HISTOPATHOLOGICAL STUDIES:[105]

The animals were sacrificed and their colon was cut into small pieces, preservedand fixed in 10% formalin for two days. The liver pieces were washed in runningwater for about 12 hrs, followed by dehydration with Isopropyl alcohol ofincreasing strength 70%, 90% and Absolute alcohol respectively for 12hrs each.

Clearing was done in two steps

- By using acetone with two changes (70% and Absolute) for 15-20min each.
- By using two changes of xylene (since xylene is a good solvent for paraffin wax).

Embedding in Paraffin by Vacuum:

Hard paraffin was melted and the hot paraffin was poured into L-shaped blocks.The colon pieces were then dropped into molten paraffin quickly and allowed tocool.

Sectioning:

The blocks were cut using microtome to get sections of thickness of 5µ. Thesections were taken on a micro-slide on which egg albumin (sticking substance)was applied. The sections were then allowed to remain in an oven at 60˚C for 1hr. Paraffin melts and egg albumin denatures, thereby fixing tissues to the slide.

Staining:

Eosin is an acid stain. Hence it stains all the cell constituents which are basic innature to pink color e.g. RNA, Cytoplasm. Haematoxylin is a basic stain whichstains all the acidic cell components blue e.g. DNA in nucleus.

Statistical analysis

All data were expressed as Mean ± SEM. The statistical Significance betweengroups were compared using one way ANOVA, followed by Dunnett's (multiplecomparison test).

RESULTS

Table 2: Percentage yield of PGPE was found to be 11.32 % as shown in table 2

Solvent	Color and consistency	Method	Percentage yield
Water and ethanol	Greenish brown	Hot Percolation	11.32 %

5.1 Preliminary phytochemical screening

Results of the preliminary phytochemical investigation of HAPP peels are shown in the **Table 3.**

Table 3: Preliminary phytochemical screening of PGPE

Sl no	Test	Result
1	Carbohydrates	+ve
2	Flavonoids	+ve
3	Glycosides	+ve
4	Steroids	+ve
5	Tannins	+ve
6	Alkaloids	-ve

5.2 DETERMINATION OF ACUTE TOXICITY

Hydro alcoholic extract of *P. granatum* fruit peel was studied for acute toxicity at dose 2000 mg/kg by p.o. route according to OECD guideline No.425. It was found to be safe up to 2000 mg/kg body wt. by oral route. There was no mortality amongst the animals and did not show any toxicity or behavioral changes. The extract found to be safe or non-toxic in rats, hence 1/10th of 2000 mg/kg and 1/5th of 2000 mg/kg were taken as low dose (200 mg/kg) and high dose (400 mg/kg) respectively.

5.3 ACETIC ACID INDUCED COLITIS IN RATS

Instillation of acetic acid (intra-rectal route) caused inflammatory reaction in the colon. The inflammation covered rectum and distal colon portion. The visible changes included ulcerated mucosa and severe epithelial necrosis.

Effect on antioxidant Parameters like MPO, LPO, CAT and GSH in acetic acid induced colitis:

From the biochemical assays, it is found that the colitis control group animals were found to be significantly altered as compared to that of normal group. MPO and LPO parameters were increased when compared to that of normal group. But GSH and CAT were decreased when compared to that of normal group.

The prophylactic treatment with sulfasalazine (100 mg/kg) showed extremely significant ($P<0.001$) decrease in MPO and LPO, and increase in GSH and CAT when compared to that of colitis control group.

The animals treated with **PGPE-200** showed moderately significant ($p<0.01$) decrease in MPO and LPO, and moderately significant ($p<0.01$) increase in CAT and less significant ($p<0.05$) increase in GSH when compared to that of colitis control group.

The animals treated with **PGPE-400** showed extremely significant ($P<0.001$) decrease in MPO and LPO, and extremely significant ($P<0.001$) increase in CAT, and a moderately significant ($p<0.01$) increase in GSH when compared to that of colitis control. Results are summarized in **Table: 4**

From the histopathology result **(Fig. 17)**, it is clear that in the normal colon (A), there was no tissue injury, epithelial damage and neutrophil infiltration. But in acetic acid induced colitis group (B), there was severe epithelial damage, tissue injury and neutrophil infiltration. In

case of PGPE 200, test low dose (C), there was moderate epithelial damage, tissue injury and neutrophil infiltration, and in PGPE 400, test high dose (D), there was less epithelial damage, tissue injury and neutrophil infiltration. In sulfasalazine treated group (E) there was very less epithelial damage, tissue injury and neutrophil infiltration.

Table 4: Effect of sulfasalazine and PGPE on MPO, LPO, CAT, and GSH in acetic acid induced colitis.

Treatment	MPO Abs at 460 nm	LPO Abs at 535 nm	CAT Abs at 560 nm	GSH Abs at 412 nm
Normal	0.08±0.01	0.08±0.03	0.48±0.05	0.46±0.05
Acetic acid control	0.46±0.08	0.36±0.01	0.17±0.02	0.13±0.08
Standard (sulfasalazine 100 mg/kg)	0.12±0.04 ***	0.17±0.02 ***	0.40±0.08 ***	0.34±0.02 ***
PGPE 200 mg	0.38±0.02 **	0.30±0.01 **	0.22±0.07 **	0.24±0.02 *
PGPE 400 mg	0.22±0.04 ***	0.24±0.08 ***	0.29±0.01 ***	0.28±0.06 **
All the values are in absorbance ± mean, n= 6, *p<0.05 **p<0.01, ***p<0.001 one way ANOVA followed by Dunnett's t test compared to positive control.				

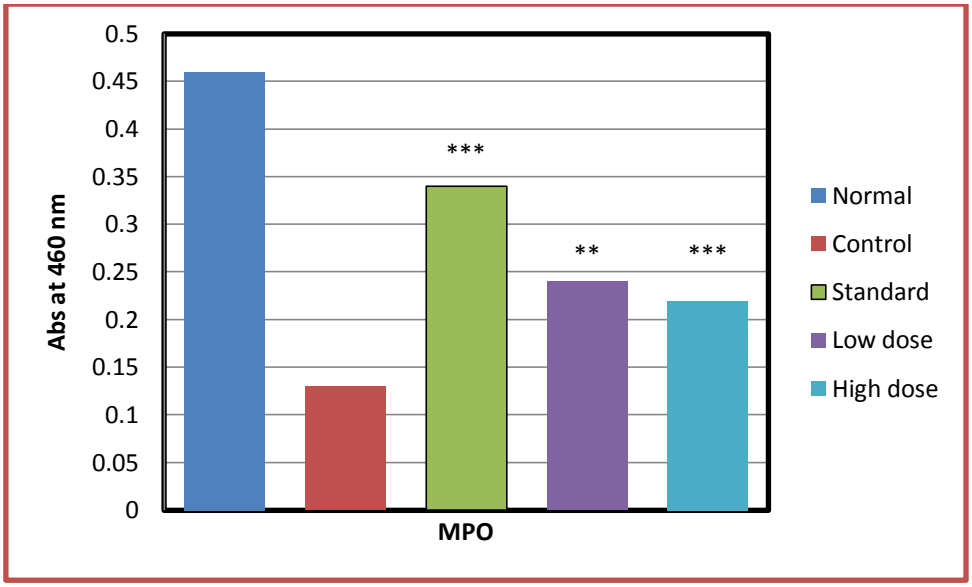


Fig 13: Effect of sulfasalazine and PGPE on MPO in acetic acid induced colitis:

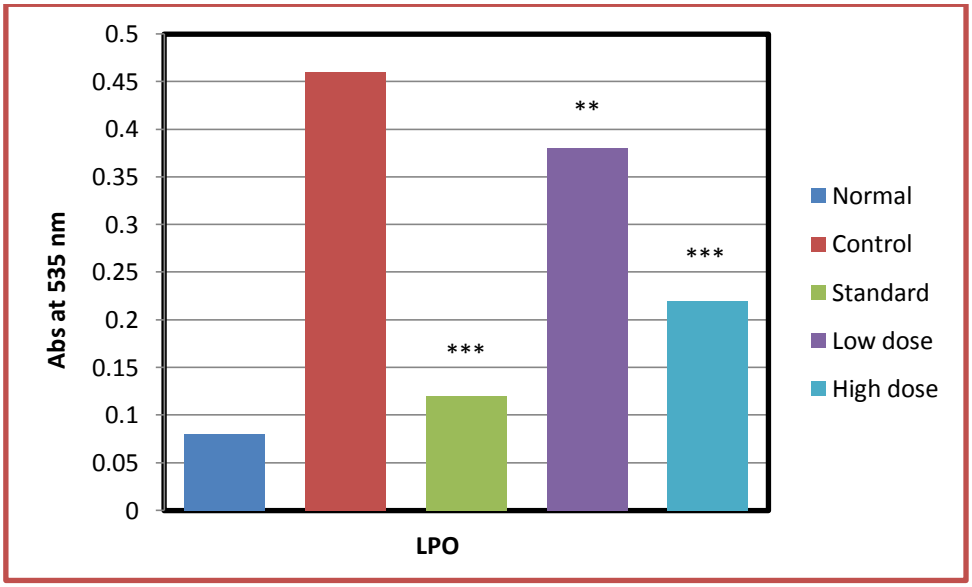


Fig 14: Effect of sulfasalazine and PGPE on LPO in acetic acid induced colitis:

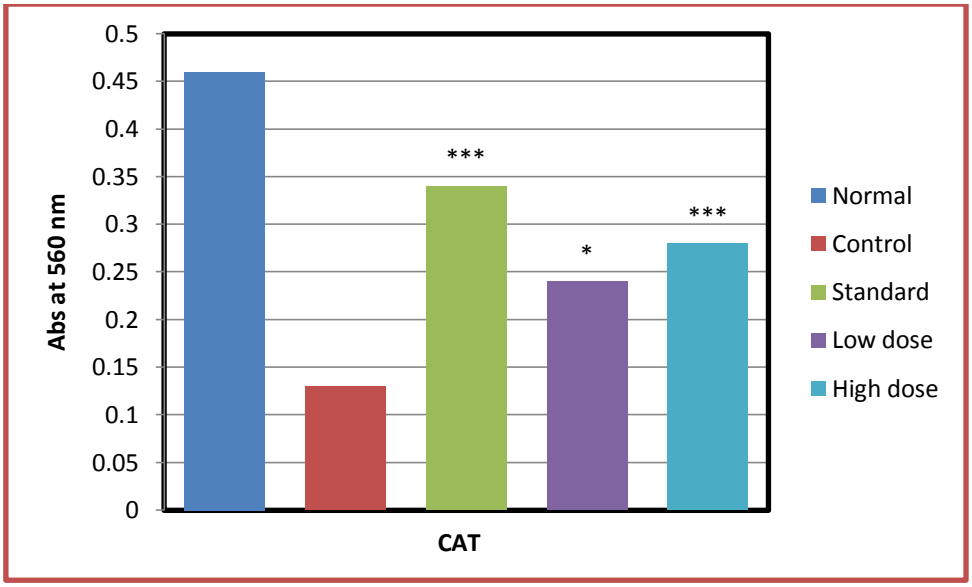


Fig 15: Effect of sulfasalazine and PGPE on CAT in acetic acid induced colitis.

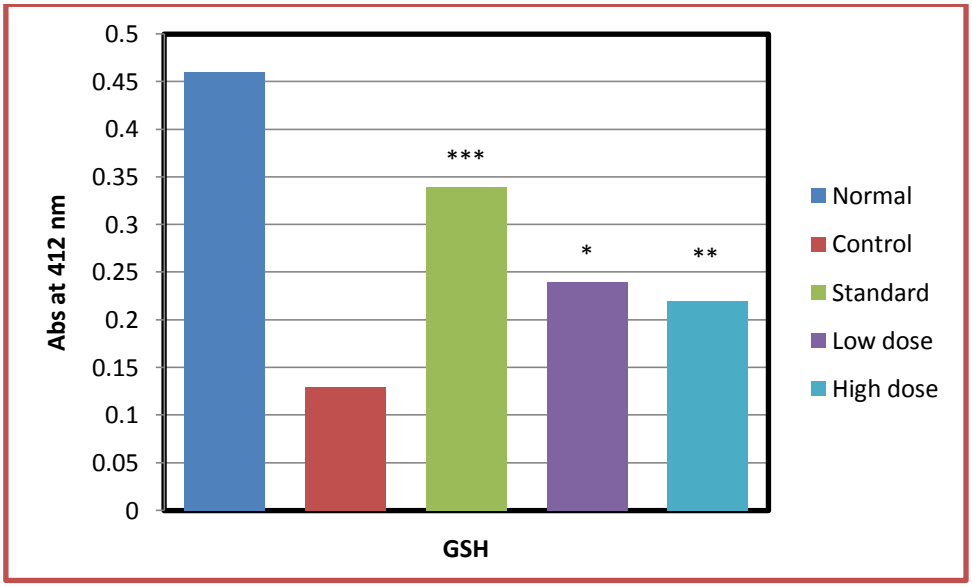
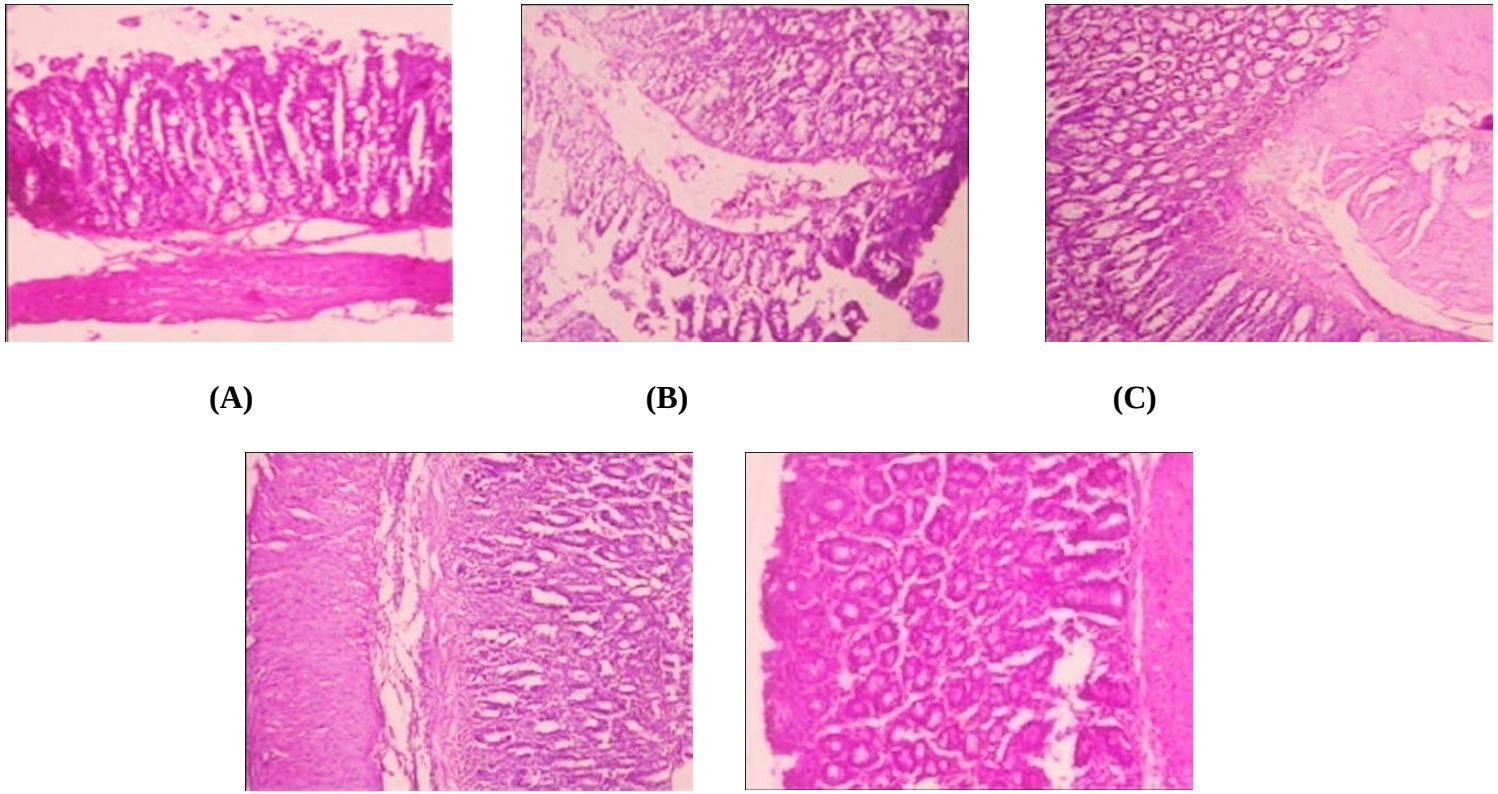


Fig 16: Effect of sulfasalazine and PGPE on GSH in acetic acid induced colitis.

Fig. 17: **Effect of Sulfasalazine and PGPE on colon histology in acetic acid induced colitis.**



(A) **(B)** **(C)**

(D) **(E)**

A) Normal control: normal texture of colon tissue, B) Possitive control (Acetic acid treated): Severe tissue damage and neutrophil infiltration. C) Low dose (PGPE-200): Moderate tissue damage and neutrophil infiltration. D) High dose (PGPE400): Mild tissue damage and neutrophil infiltration. E) Standard (Sulfasalazine treated): Less tissue damage and neutrophil infiltration.

5.4 TRINITROBENZENE SULFONIC ACID (TNBS) INDUCED COLITIS

From the biochemical assays, it is found that the colitis control group animals were found to be significantly altered as compared to that of normal group. MPO and LPO parameters were increased when compared to that of normal group. But GSH and CAT parameters were decreased when compared to that of normal group.

The prophylactic treatment with standard drug **sulfasalazine** (100 mg /kg), showed extremely significant ($p<0.001$) decrease in MPO and LPO parameters, increase in GSH and CAT parameters.

The animals treated with **PGPE-200** showed less significant ($p<0.05$) decrease in MPO and LPO parameters, a moderately significant ($p<0.01$) increase in CAT and less significant ($p<0.05$) increase in GSH.

The animals treated with **PGPE-400** Showed a moderately ($p<0.01$) significant decrease in MPO and LPO parameters, an extremely significant ($p<0.001$) increase in CAT parameter and a moderately significant ($p<0.01$) increase in GSH parameter.

From the histopathology result **(Fig. 22)**, it is clear that in the normal colon (A), there was no tissue injury, epithelial damage and neutrophil infiltration. But in acetic TNBS colitis group (B), there was severe epithelial damage, tissue injury and neutrophil infiltration. In case of PGPE 200, test low dose (C), there was moderate epithelial damage, tissue injury and neutrophil infiltration, and in PGPE 400, test high dose (D), there was less epithelial damage, tissue injury and neutrophil infiltration. In sulfasalazine treated group (E) there was very less epithelial damage, tissue injury and neutrophil infiltration

Table 5: Effect of sulfasalazine and PGPE on MPO, LPO, CAT, and GSH in TNBS induced colitis

Treatment	MPO Abs at 460 nm	LPO Abs at 535 nm	CAT Abs at 560 nm	GSH Abs at 412 nm
Normal	0.17±0.008	0.21±0.03	0.62±0.02	0.70±0.01
TNBS control	0.56±0.017	0.65±0.01	0.21±0.01	0.47±0.02
Standard (sulfasalazine 100 mg/kg)	0.30±0.05 ***	0.28±0.34 ***	0.52±0.04 ***	0.66±0.01 ***
PGPE 200 mg	0.42±0.04 *	0.55±0.01 *	0.36±0.07 **	0.49±0.02 *
PGPE 400 mg	0.35±0.02 **	0.51±0.08 **	0.44±0.02 ***	0.60±0.08 **
All the values are in absorbance ± mean, n= 6, *p<0.05 **p<0.01, ***p<0.001 one way ANOVA followed by Dunnett's test compared to positive control.				

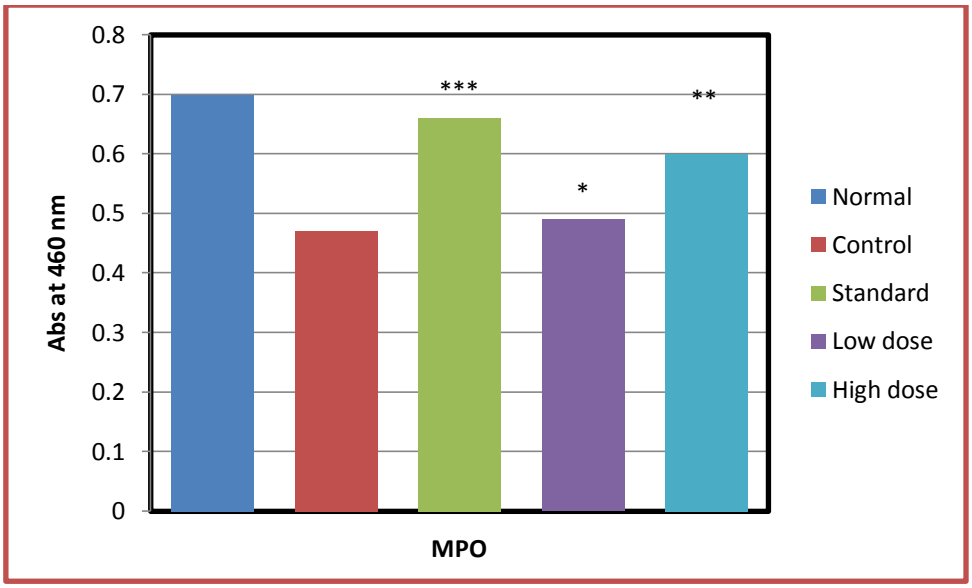


Fig 18: Effect of sulfasalazine and PGPE on MPO in TNBS induced colitis.

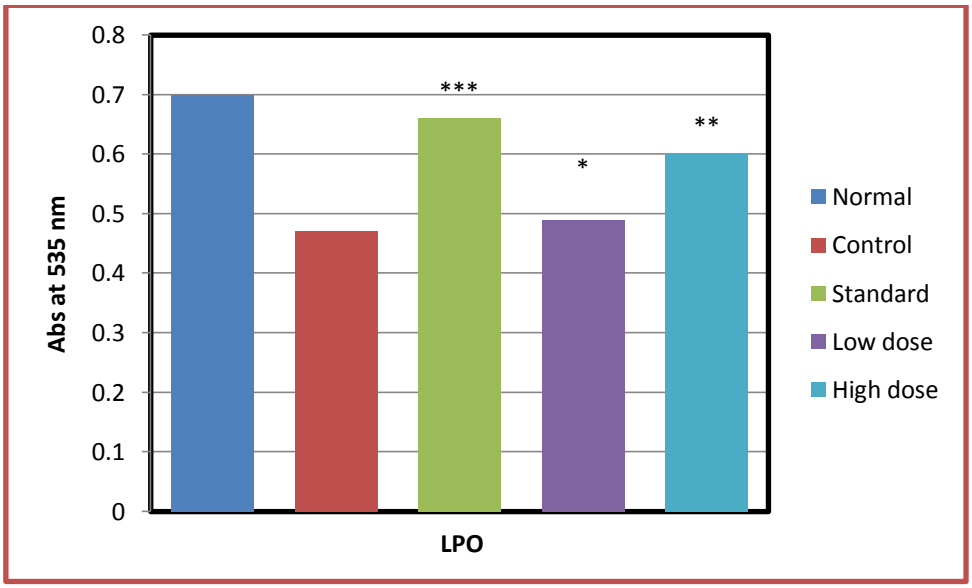


Fig 19: Effect of sulfasalazine and PGPE on LPO in TNBS induced colitis.

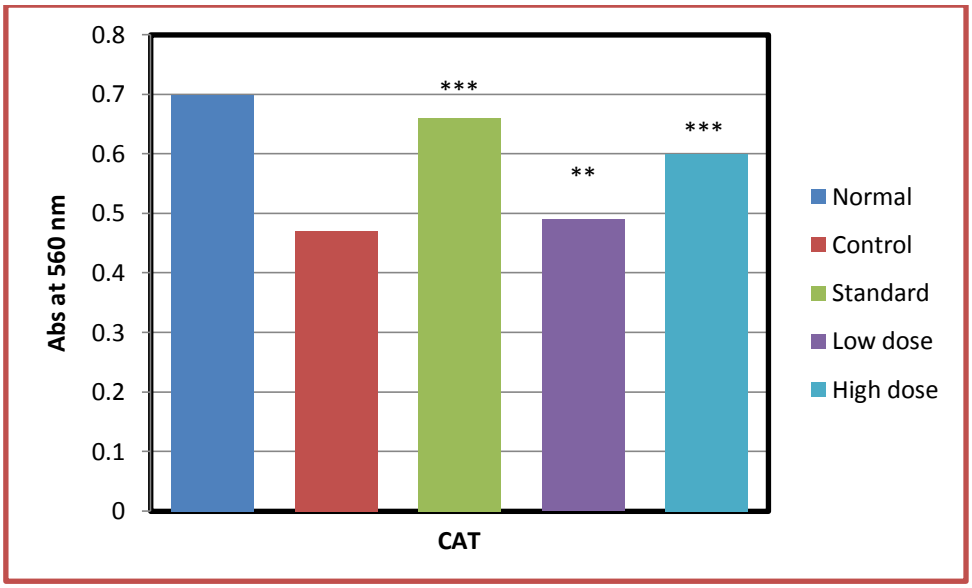


Fig 20: Effect of sulfasalazine and PGPE on CAT in TNBS induced colitis.

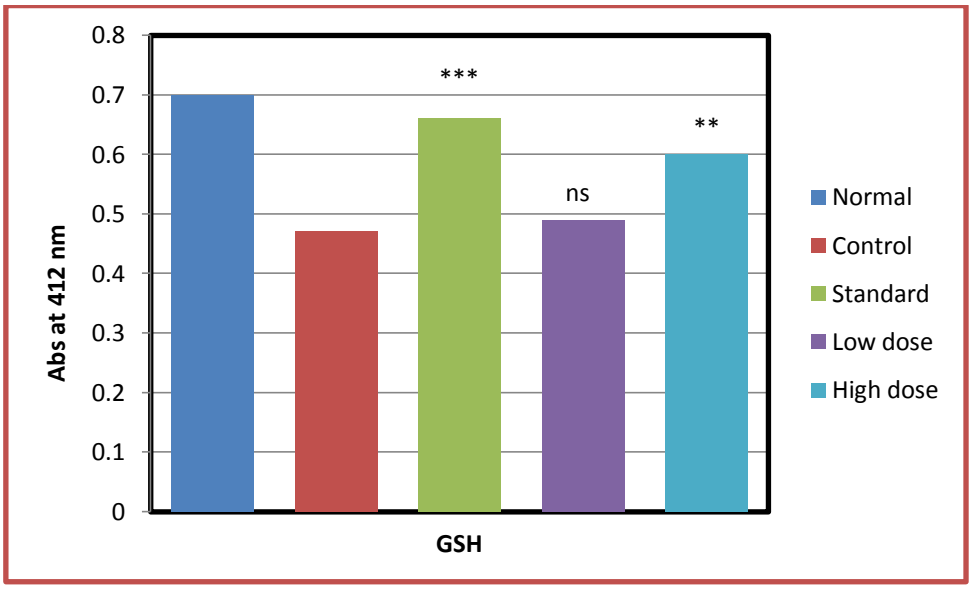
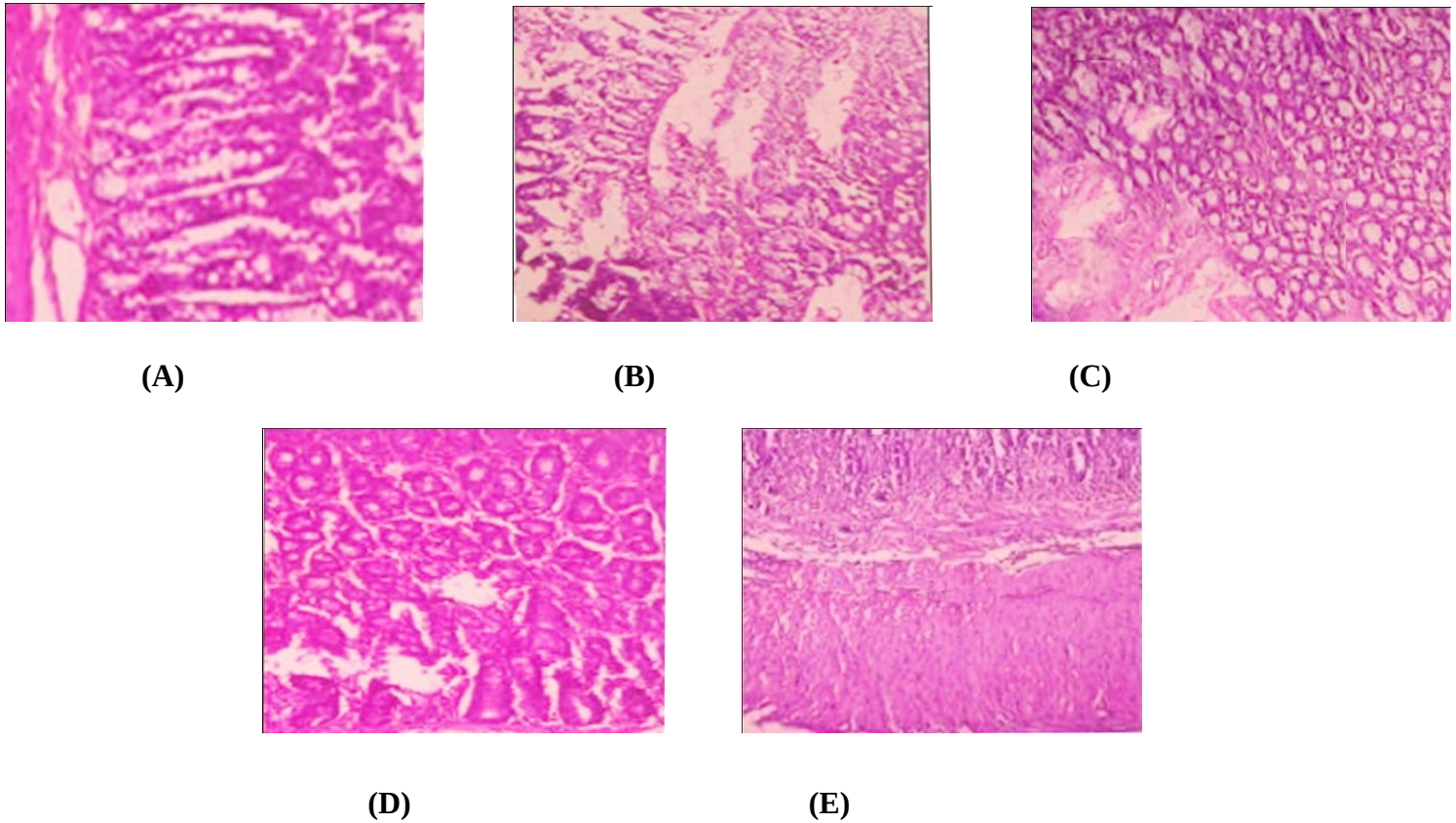


Fig 21: Effect of sulfasalazine and PGPE on GSH in TNBS induced colitis.

Fig .22: Effect of Sulfasalazine and PGPE on colon histology in TNBS induced colitis.



(A) **(B)** **(C)**

(D) **(E)**

A) Normal control: normal texture of colon tissue, B) Possitive control (Acetic acid treated): Severe tissue damage and neutrophil infiltration. C) Low dose (PGPE-200): Moderate tissue damage and neutrophil infiltration. D) High dose (PGPE400): Mild tissue damage and neutrophil infiltration. E) Standard (Sulfasalazine treated): Less tissue damage and neutrophil infiltration.

DISCUSSION

The present study was undertaken to verify the protective effect of hydro-alcoholic extract of *P. granatum* fruit peels in colonic ulcer induced by various toxic substances like acetic acid and TNBS.

The models used for the study of inflammatory bowel disease (IBD) are acetic acid induced ulcerative colitis and TNBS induced ulcerative colitis. Intra rectal instillation of acetic acid in rats affected only the distal colon portion. The inflammation was not transmural. Massive necrosis of mucosal and sub mucosal layers were observed. This model shares many of the histological features of ulcerative colitis in human beings including mucosal edema, neutrophil infiltration of the mucosa and sub mucosal ulceration[106].

The mechanism by which acetic acid produces inflammation appears to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons causing intracellular acidification that most likely account for the observed epithelial injury. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipooxygenase pathways and generation of inflammatory mediators like prostaglandins and leukotrienes. Excess production of reactive oxygen metabolites e.g. superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives such as N-chloramines are detected in inflamed mucosa and may be pathogenic in IBD. Also, there is an increase in proinflammatory cytokine TNF -α production in colonic mucosa after acetic acid instillation[107].

Colitis can be induced in susceptible mouse strains by intra rectal instillation of the haptenating substance TNBS. Massive necrosis of mucosal and sub mucosal layers were observed. This model showed many of the histological features of ulcerative colitis in human

beings including mucosal edema, neutrophil infiltration of the mucosa and sub mucosal ulceration. TNBS dissolved in ethanol with or without a skin pre-sensitation step. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. Histological features and an elevated production of TH2 cytokines (IL-4, IL-5 and IL-13) of unstimulated and αCD3/αCD28-stimulated lamina propria T cells are in these rats, in some aspects, similar to characteristics that have been observed in human UC[92].

The treatment with hydro-alcoholic extract of *P. granatum* fruit peel has showed a decrease in the macroscopic scores for the inflammation. Since the intestine is in a constant state of controlled inflammation, thus amplification of the inflammatory response activates infiltration of inflammatory cells that triggers pathological responses and symptoms of IBD[108].

Myeloperoxidase (MPO) is an enzyme mainly found in azurophilic granules of neutrophils. It can serve as a good marker of inflammation, tissue injury and neutrophil infiltration in gastrointestinal tissues. It was increased with acetic acid and TNBS. Pretreatment with *P. granatum* peels extract exhibits decrease in poly morphonuclear infiltration demonstrated by significant reduction in MPO activity. Oxidative damage may represent crucial pathogenic factor in IBD because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species[109].

MDA is considered as an important indicator of lipid peroxidation[110], which is found to be increased in rats treated with acetic acid and TNBS. This might be due to lipid peroxidation. Rat pretreatment with *P. granatum* extract showed protection against lipid peroxidation characterized by significant decrease in MDA level.

Oxidative stress is believed to play a key role in the pathogenesis of IBD-related intestinal damage[111]. Intestinal mucosal damage in the IBD is related to both increased free radical production and a low concentration of endogenous antioxidant defence[112].

The antioxidant enzyme, catalase is the first line defensive enzyme against free radicals[113]. In the present study, it is decreased with acetic acid and TNBS treated groups, where as in *P. granatum* peel extract treated animals antioxidant parameter (CAT) is significantly increased. This shows that the *P. granatum* peel extract can reduce reactive free radicals that might cause oxidative damage to the tissues.

GSH is a natural antioxidant present in the body, which is found to be decreased in rats treated with acetic acid and TNBS[114]. In the present study, it was observed that pretreatment with *P. granatum* peel extract exhibits elevated GSH level.

Histopathology examination of drug treated group revealed less damage compared to colitis control group. A dose dependant decrease in MPO, MDA level is monitored, and dose dependant increase in GSH and CAT were also monitored. All these observations support the findings that the hydro-alcoholic extract of *P. granatum* peel is able to offer significant protection in both the models. The sulfasalazine treatment has shown significant protection in both the animal models studied.

The major classes of pomegranate phytochemicals are polyphenols that predominate in fruits[95]. Pomegranate polyphenols include flavonoids, condensed tannins and hydrolysable tannins. Hydrolysable tannins (HTs) are found in the peels (rind, husk, or pericarp), membranes and piths of the fruit. HTs are predominant polyphenols found in pomegranate juice and account for 92% of its antioxidant activity[96].

In the preliminary phytochemical screening, it was observed that the hydro-alcoholic extract of *P. granatum* peels contained polyphenols like glycosides, flavonoids, tannins and steroids. Presence of these polyphenols in the extract might attributed to their antioxidant properties, in turn responsible for protective effect against colitis

On the basis of above experimental data it can be concluded that the hydro-alcoholic extract of *P. granatum* fruit peel might be useful in treating ulcerative colitis in humans.

CONCLUSION

The present study concludes that hydro alcoholic extract of *P. granatum* peel witnessed a significant dose dependent ulcerative colitis protective activity in both acetic acid and TNBS induced experimental models.

The ulcerative colitis protective activity was found to be more significant in high dose PGPE 400 mg/kg compared to low dose PGPE 200 mg/kg. The *P. granatum* peel extract for ulcerative colitis protective activity was comparable with standard drug sulfasalazine.

The hydro alcoholic extract of *P. granatum* fruit peel showed a dose dependant decrease in biomarkers like MPO and MDA and increase in GSH and CAT. From the histopathological study, it is clear that the *P. granatum* fruit peel have reversed the damage produced by acetic acid and TNBS.

The ulcerative colitis protective activity of *P. granatum* in the experimental models may be due to the presence of polyphenols like flavonoids, tannins, etc, which are attributed to potential anti oxidant activity.

The exact mechanism behind ulcerative colitis protective activity of hydro alcoholic extract of *P. granatum* is still unclear, further study is needed to assess exact mechanism and characterize the active principles responsible for ulcerative colitis protective activity.

SUMMARY

The hydro-alcoholic extracts of *P. granatum* peels were evaluated for ulcerative colitis protective activity using animal models such as acetic acid induced colitis and TNBS induced colitis.

Extraction and preliminary phytochemical investigation of *P. granatum* peels have revealed the presence of carbohydrates, flavonoids, glycosides, tannins and steroids.

Acute toxicity study was performed to find out the safe dose according to OECD guidelines 425 and hydro alcoholic extract of *P. granatum* peel was found to be safe at 2000 mg/kg.

Ulcerative colitis protective activity was studied against acetic acid (3 %, 2 ml), TNBS (I ml in 50 % ethanol) induced ulcerative colitis, using Sulfasalazine (100 mg/ kg) body weight as standard reference. Biochemical parameters such as MPO, LPO, CAT, GSH and histopathology of colon tissue were also studied.

Reported activity from the study showed a significant dose dependent ulcerative colitis protective activity for PGPE 200 and PGPE 400 is correlated with decrease in biomarkers like MPO and MDA and increase of GSH and CAT which showed anti oxidant activity.

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